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FRESHWATER ASSAY USING SOIL ELUATES AS SAMPLE MATERIAL

(Single Laboratory Evaluation)

by

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16. ABSTRACT

The Chlorophyta assay, which uses soil as sample material, has been a useful bioassessment technique for screening hazardous waste site problems. An eluate is prepared from a 125-gram soil sample and then diluted into three separate concentrations prior to being tested using Selenastrum capricornutum. The work repprted here has attempted to determine the procedure's capability for data quality, to provide a basis for deciding whether the assay merits collaborative testing, and to define more clearly the method's potential for inclusion as part of an operational monitoring network. The soil used for most of this evaluation was a homogenized clay loam. Soil containing either sodium fluoride or 2,4-dichlorophenol was tested using the algal assay, and several tests were conducted to confirm the dose/response curve using the positive control compound, zinc chloride. A known test response also was established using a white silica sand spiked with sodium fluoride. The known test response provides a value with a fairly high standard error, which, in some ways, probably detracts from the future application of this technique when accuracy and/or systematic error estimates are needed. Results revealed an apparently poor capability for sensitivity and a somewhat limited range of reliable measurement. The procedure, however, will definitely detect the presence of certain chemicals and, thus, can bbe used effectively as a screening technique.

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NOTICE

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FOREWORD

As environmental controls become more costly to implement and the penalties of judgment errors become more severe, environmental quality management requires more efficient analytical tools based on greater knowledge of the environmental phenomena to be managed. As part of this Laboratory's research on the occurrence, movement, transformation, impact, and control of environmental contaminants, the Biology Branch evaluates procedures for assessing environmental toxicity at hazardous waste sites.

Biological methods used in monitoring contaminants at waste sites must be capable of providing repeatable results. Consequently, it is necessary to determine each procedure's capability for producing high quality data in single laboratory use and in multiple laboratory applications. This report presents a single laboratory's evaluation of a freshwater assay method based on a procedure developed by EPA's Environmental Research Laboratory, Corvallis OR, for the bioassessment of hazardous waste sites. The assay method has been tested at actual waste sites and has been found to be one of the better screening techniques for detecting the presence of potentially hazardous compounds.

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ABSTRACT

The Chlorophyta assay, which uses soil as sample material, has been an effective bioassessment technique for hazardous waste site problems. An eluate is prepared from a 125 gram soil sample and then diluted into three separate concentrations prior to being tested using Selenastrum capricornutum. The current effort has attempted to determine the procedure's capability for data quality, to provide a basis for deciding whether or not the assay merits collaborative testing, and to more clearly define the method's potential for inclusion as part of an operational monitoring network.

The soil used for most of this evaluation was a homogenized clay loam (characterized as being 22% sand, 51% silt, and 27% clay). Samples were chemically analyzed using ICAP; GC/MS; and Kjeldahl nitrogen techniques to confirm that a reasonably homogenized soil had been selected for the evaluation. Soil containing either sodium fluoride or 2,4-dichlorophenol was tested using the algal assay and several tests were conducted to confirm the dose/response curve using the positive control compound zinc chloride. A known test response was also established using a white silica sand spiked with sodium fluoride.

Determinations for precision, sensitivity, limits of reliable measurement, and a known test response are included in the report. The ruggedness test results and the subsequently revised method protocol are also included. While considerable progress was made toward a standardization of this procedure, some difficulties remain when one considers a continuation to collaborative testing. The known test response provides a value with a fairly great standard error which, in some ways, probably detracts from the future application of this response for use in accuracy and/or systematic error estimates. The current evaluation also revealed an apparently poor capability for sensitivity and a somewhat limited range of reliable measurement. However, the procedure will definitely detect the presence of pollutant compounds and, by design, the procedure is used only as a screening technique. Consequently, the observed method sensitivity should not be considered as being detrimental to further method standardization or to future application as part of a monitoring network.

The greatest problem confronting a continuation to collaborative testing may be the recurring lack of consensus agreement noted among knowledgeable biologists for several phases of the method protocol. However, many frequently used procedures have never enjoyed total consensus agreement and various organizations such as the Association of Official Analytical Chemists (AOAC) and the American Society for Testing and Materials (ASTM) frequently publish method revisions as well as interim protocols to allow for ongoing improvements. The authors of this report recommend that the current algal assay be collaboratively tested as a next step toward more frequent application of the procedure for hazardous waste monitoring.

CONTENTS

Notice ii
Foreword iii
Abstract iv
List of Tables vi
List of Figures viii
Consensus Review Group ix

Introduction 1
Procedure 2
  Single Laboratory Evaluation 2
  Consensus Review 2
  Freshwater Assay Using Soil Eluates 3
    Soil Preparation 3
    Eluate Dilutions 4
    Cell Culture 4
    Nutrient Composition 4
    Positive Control 4
  Sample Material 8
    Clay Loam Soil 8
    Chemical Analysis 10
Results and Discussion 14
  Ruggedness 14
  Precision 17
  Response to Organic Compound 18
  Method Sensitivity 20
  Limits of Reliable Measurement 20
  Accuracy 22
Conclusions 23
Bibliography 26

Appendix A - Algal Cell Concentrations 28
Appendix B - Method Protocol 41
Appendix C - Consensus Review Criticisms 62

LIST OF TABLES

Table		Page
I.	Composition of Algal Growth Media	7
II.	Chlorophyta Response to Various Concentrations of Zinc Chloride	8
III.	Series of Algal Assays Using 128 µg/l of Zinc Chloride	9
IV.	Series of Algal Assays Using 74 µg/l of Zinc Chloride	10
V.	Element Analysis of Soil Subsamples Taken from the Homogenized Soil Collection Used for the Chlorophyta Evaluation	11
VI.	Approximate Transfer of Sodium Fluoride from Soil to Eluate During the Chlorophyta Assay Evaluation	12
VII.	Transfer of Soil Elements to the Subsequently Prepared Eluate	13
VIII.	Experimental Design for a Seven Variable Ruggedness Test	15
IX.	Test Variables Used to Evaluate Ruggedness of Chlorophyta Procedure	16
X.	Relative Effect of Chlorophyta Protocol Variations on Final Test Result	18
XI.	Chlorophyta Assay's Single Laboratory Capability for Precision	19
XII.	Assay Response Using Soil Samples Containing Either Sodium Fluoride or 2,4-Dichlorophenol	21
XIII.	Response of Chlorophyta Assay Procedure to Various Soil Concentrations of Sodium Fluoride	22
XIV.	Determinations to Establish a Known Response to a Reference Sample	24

Appendix Table

A-I.	Algal Cell Concentration Determined at 96 Hour Interval in Response to Eluate from 80.0 µg/g Dichlorophenol Soil Sample	29
A-II.	Algal Cell Concentration Determined at 96 Hour Interval in Response to Eluate from 4.5 mg/g Sodium Fluoride Soil Samples	30
A-III.	Algal Cell Concentration Determined at 96 Hour Interval in Response to Eluate from 5.6 mg/g Sodium Fluoride Soil Samples	31
A-IV.	Algal Cell Concentration Determined at 96 Hour Interval in Response to Eluate from 6.4 mg/g Sodium Fluoride Soil Samples	32

LIST OF TABLES (continued)

Table		Page
A-V.	Algal Cell Concentration Determined at 96 Hour Interval in Response to Eluate from 7.2 mg/g Sodium Fluoride Soil Samples	33
A-VI.	Algal Cell Concentration Determined at 96 Hour Interval in Response to Eluate from 8.7 mg/g Sodium Fluoride Soil Samples	34
A-VII.	Algal Cell Concentration Determined at 96 Hour Interval in Response to Eluate from 8.9 mg/g Sodium Fluoride Soil Samples	35
VIII.	Algal Cell Concentration Determined at 96 Hour Interval in Response to Eluate from 17.8 mg/g Sodium Fluoride Soil Samples	36
A-IX.	Algal Cell Concentration Determined at 96 Hour Interval in Response to Eluate from 26.7 mg/g Sodium Fluoride Soil Samples	37
A-X.	Algal Cell Concentration Determined at 96 Hour Interval in Response to Eluate from 35.6 mg/g Sodium Fluoride Soil Samples	38
A-XI.	Algal Cell Concentration Determined at 96 Hour Interval in Response to Eluate from 48.0 mg/g Sodium Fluoride Soil Samples	39
A-XII.	Algal Cell Concentration Determined at 96 Hour Interval in Response to Eluate from 60.0 mg/g Sodium Fluoride Soil Samples	40
B-I.	Composition of Algal Growth Media	54
B-II.	Chlorophyta Response to Various Concentrations of Zinc Chloride	59
B-III.	Series of Algal Assays Using 128 µg/l of Zinc Chloride	60
B-IV.	Series of Algal Assays Using 74 µg/l of Zinc Chloride	61

Note: Some of the material in the method procedure (Appendix B) has been included in the evaluation report. This previously mentioned information includes material presented in Appendix B, Tables I through IV.

FIGURES

Number		Page
1	Example of algal growth	5

CONSENSUS REVIEW GROUP

The original method protocol was prepared by ERL-Corvallis (Miller, Greene, and Peterson, 1986). The consensus review and/or the final evaluation review group was composed of Joseph Greene (EPA-Corvallis); Richard Kimerle (Monsanto Corporation); Randall Miles (University of Missouri, Department of Agronomy); Woodrow Wang (Illinois State Water Survey); Donald Miles (University of Missouri, Department of Biology); Michael Lewis (Battelle Columbus); James Hamala (Technology Applications, Inc.); Sandra Bird (EPA-Athens); William Sutton (EPA-Athens); William van der Schalie (Army Biomedical Research and Development Laboratory); and William Lower (University of Missouri, Environmental Trace Substances Center).

INTRODUCTION

Assessing current and potential problems at uncontrolled hazardous waste sites has been very difficult because of several factors including (1) the complex chemical mixtures found at most sites and (2) the fact that toxicity and environmental transport data are limited for many of the compounds present at the sites, especially for those that are by-products of organic synthesis (rather than commercial chemicals). Biological tests can provide several unique and important benefits when used as part of waste site monitoring programs. Among others, they can be used to predict the potential hazard posed by unknown mixtures of chemicals, they can be used to identify the bioactive fractions of unknown chemical mixtures, and similarly, they can detect toxic substances not normally reported by standard chemical analysis.

Time will not permit intensive chemical monitoring at every known hazardous waste site, nor will it permit intensive monitoring at all locations (geographic areas) surrounding a given hazardous waste site. Methods are needed that can assist in (1) selecting specific geographic areas for intensive monitoring, (2) identifying specific waste sites that pose the greatest potential hazard, or (3) determining the most hazardous contaminants at a particular waste site. Such information would be of benefit in planning remedial action strategies and in making decisions about which sites should have the highest priority for remedial action.

If biological methods are to be used as part of the waste site monitoring effort, the selected procedure(s) must be capable of providing repeatable results. Consequently, it is necessary to determine the procedure's capability for data quality in the single laboratory and, by way of a collaborative test, in multiple laboratories. The current effort concerns an evaluation of this modified freshwater assay in a single laboratory. The assay is based on a procedure developed by EPA's Environmental Research Laboratory, Corvallis, OR, for the bioassessment of hazardous waste sites. The assay method has been tested at actual waste sites and found to be one of the better screening techniques for detecting the presence of potentially hazardous compounds. Since an aqueous eluate is acquired initially from the original soil sample, and since this eluate is filtered and diluted prior to toxicity testing, the Chlorophyta procedure does not address non-water soluble chemicals nor does it test for the presence of volatile compounds. In addition, procedures associated with the collection of soil samples (e.g., representative sampling of a waste site area) are not addressed. Based on the ecological importance of unicellular algae, however, this relatively simple technique can provide an environmental hazard assessment for those chemicals that are, in fact, the compounds most likely to be transported to surface and ground waters.

The algal assay component of this procedure was basically derived from a freshwater assay that has been traditionally used to determine growth inhibition and stimulation. Several previous efforts have been directed toward the standardization of this original technique (International Organization for Standardization, 1987; Standard Methods, 1985; ASTM, 1985; Federal Register, Vol. 50, #188, 1985).

The current single laboratory effort will establish the data quality that can be achieved within a single laboratory. The current evaluation should also provide a basis for deciding whether or not this modified algal procedure merits

collaborative testing and should more clearly define the assay's potential for inclusion as part of an operational monitoring network.

PROCEDURE

Single Laboratory Evaluation

Phases of the single laboratory test include identification of procedural variables that must be carefully controlled (ruggedness testing), evaluation of method sensitivity, identification of the limits of reliable measurement, evaluation of systematic error (bias), and identification of method precision and accuracy.

A complete protocol, for the method being evaluated, must be received by the evaluating laboratory prior to test initiation. All method requirements and procedural instructions should be clearly presented. It obviously is important that these written instructions be technically correct, complete, and as unambiguous as possible. However, the laboratory conducting the single laboratory test is not usually responsible for the actual method itself. Before the single laboratory test begins, the evaluating laboratory must review the method protocol and make notations where ambiguous statements are made or where more detail is needed. Ambiguous statements must be clarified before the evaluation begins. It also is necessary that the written protocol represent (or at least approach) a consensus procedure. The evaluating laboratory would then strictly follow the method procedures and method requirements (experimental conditions, reagents, laboratory equipment, storage of samples, maintenance of experimental organisms, blanks, standards, replicates, etc.) as they are written in the protocol.

Ten successive analyses (i.e., acquiring ten valid responses by following the method protocol) are suggested for several phases of the single laboratory test. The method precision determination, for example, requires that ten successive independent analyses be conducted on the same sample material. Multistage calculations to determine the required number of analyses might be conducted during the single laboratory test as more information becomes available on the expected variance. However, 10 analyses will allow the test laboratory to estimate the standard deviation to within 45 percent of its true value (at a 95 percent confidence interval). The single laboratory test cost will increase rapidly as more of these successive analyses are required because each additional value must represent a valid test response and therefore will include whatever quality control analyses (blanks, replicates, etc.) are required in the original method protocol to insure a valid test response.

The single laboratory test cannot really evaluate a method's scope of applicability, i.e., the array of sample types or environmental situations for which the method would be able to provide useful data. However, the single laboratory test should include various test samples having a concentration range, in the same sample matrix, that would be encountered if the method was being used routinely for its intended purpose. Chemical composition of all sample material must be verified during the single laboratory test.

Consensus Review

Soils and sediments contaminated with various phenols and aromatic hydrocarbons have already been included among the sample types previously tested

using the Chlorophyta assay. In the case of the current evaluation, 40 or more preliminary tests were conducted using soil samples individually spiked with various toxic organic compounds (different concentrations of commercially available herbicides). These compounds had a known toxicity to several fish and mammalian species and, as expected, also produced a definite response when using the algal assay. This preliminary effort also allowed the laboratory personnel to become thoroughly familiar with the assay procedure, to recommend revisions or clarifications for the method protocol, and to ensure that all supplies and testing schedules were sufficient for the planned single laboratory evaluation.

The written method protocol was then reviewed by a group of persons familiar with both this specific assay and with many of the problems encountered at uncontrolled hazardous waste sites. A consensus review is always necessary as an attempt to achieve consensus agreement prior to beginning the method standardization process. The protocol was slightly revised based on the review comments and was then reviewed a second time by the same group of scientists. However, as is often the case, complete consensus agreement for each step of the assay, was not totally achieved. (Subsequent protocol revisions are noted below.)

Freshwater Assay Using Soil Eluates

Soil Preparation

As mentioned above, results from this Chlorophyta assay ultimately are used to assess the relative toxicity of various soil samples. Based on the ecological importance of unicellular algae, the results also contribute to an ecological hazard assessment. Approximately 500 grams of soil are collected and sent to the laboratory for testing. The algal culture (Selenastrum capricornutum) is maintained by the testing laboratory and preparations of algal growth media are prepared routinely.

A blender is initially used to homogenize the soil which is then smoothed to an even layer in a ceramic pan. The soil is then air dried before a 125-gram portion is used for the assay. Any rocks or gravel present in the sample are manually removed prior to drying. The 125-gram soil sample is transferred to a 1-liter teflon container and 500 ml of deionized water are added. The teflon container then is capped and mechanically shaken. The liquid is decanted from the container and transferred to 50-ml centrifuge tubes and then centrifuged. The total amount of soil eluate recovered from the teflon container will vary somewhat between samples but at least 400 ml should be available.

Note: A protocol revision also occurred after the ruggedness test and included revisions to emphasize critical steps in the procedure. In addition, when the evaluation was completed, the method procedure was reviewed a final time along with the summarized evaluation data. Some of the original disagreements were again present. The reviewers are listed in the acknowledgements section. An argument could be presented that too few individuals actually reviewed the procedure especially when compared to the sequence of draft procedures, interim procedures, etc., developed by such groups as ASTM. Based on the resulting diversity and sometimes conflicting areas of criticism, the addition of more reviewers might not have altered the final conclusion i.e., that consensus agreement was never totally achieved for the algal assay protocol (Appendix C).

Eluate Dilutions

This leachate (soil eluate) is filtered, and the pH is adjusted prior to testing. Several preliminary filtering steps (i.e., 2.7 µm; 1.6 µm; 1.0 µm; etc.) may be required depending on the characteristics of a precipitate noted in the resulting soil eluate. After centrifugation, however, the eluate typically is transferred to a Buchner funnel that contains a 0.7-µm, nonsterile glass fiber filter and filtered under vacuum. Subsequently, the eluate is filtered through a 0.45-µm nylon filter to obtain a sterile eluate. Although the growth response of Selenastrum capricornutum is not affected over a wide range of pH values, the potential array of chemicals and soil types encountered when using the procedure occasionally will require pH alterations for the leachate. Therefore, several pH determinations are routinely conducted and, when required, pH adjustments are made using 1N sodium hydroxide and 1N hydrochloric acid.

In the next step, the soil eluate is diluted to a series of three separate concentrations using the growth media preparation. Algal cells are transferred from the stock culture to each dilution flask. A negative control also is prepared using the growth media preparation and the algal cell addition. Zinc chloride is added in addition to the growth media and algal cells when preparing the positive control. Ninety-six hours after the flasks are inoculated, the cell concentration in each flask is determined using a coulter counter.

Duplicate flasks are prepared routinely for the negative control, positive control, 80% soil eluate, 10% soil eluate, and 1% soil eluate, respectively. In the case of the positive control, separate response values are tabulated. The first step of the final calculation is to determine the eluate effect for each of the three dilutions and the second step is to extrapolate an EC_{50} value which then serves as the final test result. The eluate effect is tabulated using the initial inoculum cell count and the 96-hour cell count determined for each eluate dilution and for the negative control. The proportion of growth media used in a given dilution flask also is included in the tabulation. A positive control response also is routinely calculated for each separate assay.

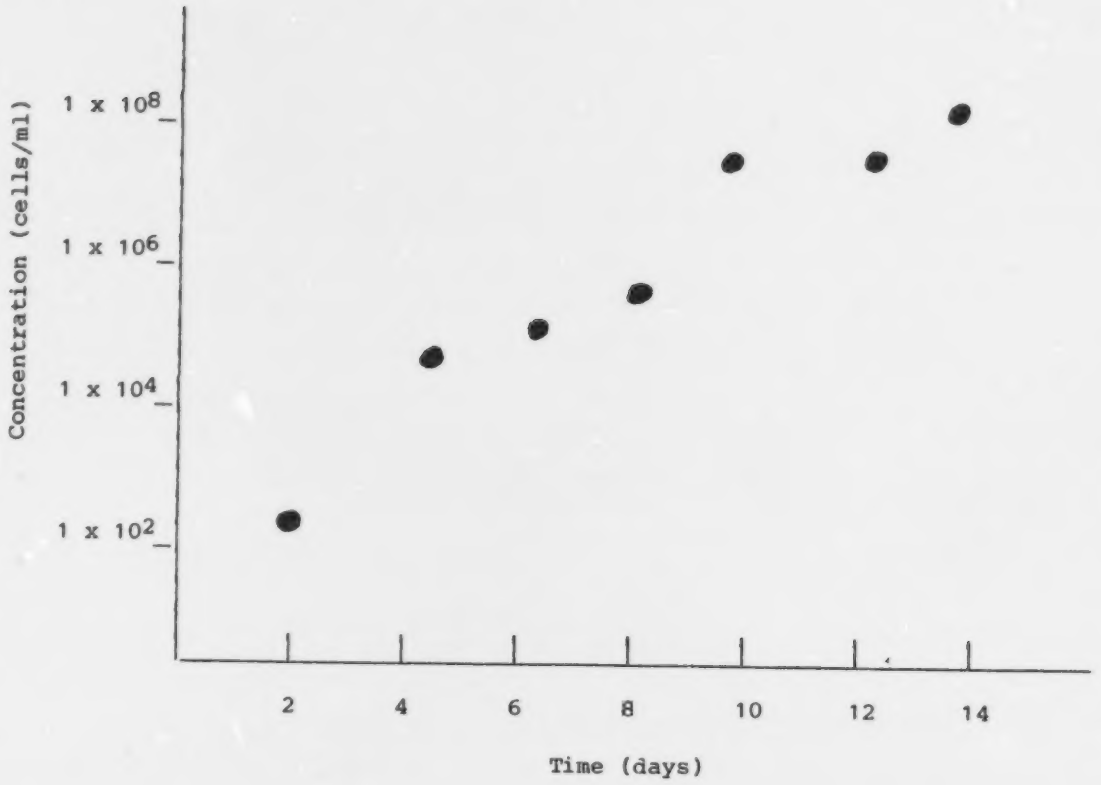
Cell Culture

A pure monoculture of algal cells is required for the assay. Stock cultures are grown in Erlenmeyer flasks and must be routinely transferred, using sterile pipettes, into flasks containing fresh growth media (Figure 1). When used for an assay, the algal cells are first transferred from the stock culture to an inoculum flask, which should contain 50 ml of previously sterilized deionized water. When the assay flasks are prepared, the cell transfer should contain a sufficient number of cells so that the resulting concentration (in the 50 ml of soil eluate plus growth medium) will be $1 \times 10^4 \pm 1 \times 10^3$ cells/ml.

Nutrient Composition

Total consensus was never really reached for the composition of nutrient media. The nutrient media used obviously will effect the algal response, but persons experienced with the assay simply do not agree on the concentration of some media components. The growth media used throughout this evaluation,

FIGURE 1. EXAMPLE OF ALGAL GROWTH FOR STOCK CULTURE OF SELENASTRUM CAPRICORNUTUM AS DETERMINED ON DAY 2,4,6,8,10,12, AND 14 AFTER ALGAL CELL ADDITION TO GROWTH MEDIA.



Note: The cell concentration in the stock culture should be approximately 4.0×10^6 cells/ml before the cells are transferred to the test flasks. Transfer to the inoculum flask is an intermediate step in the process. The resulting cell concentration (in the 50 ml of soil eluate plus growth medium) should be $1 \times 10^4 \pm 1 \times 10^3$ cells/ml. In addition, the stock culture cells must be routinely transferred to fresh medium before the culture reaches the senescent phase.

and the media composition specified in the "consensus" method protocol, is given in Table I. The medium is prepared by adding 1.0 ml from each of the Table I solutions (macronutrient and micronutrient) to 900 ml of sterilized deionized water. The final volume is taken to 1 liter.

Positive Control

Zinc chloride was used as the positive control compound but many other chemicals could undoubtedly be selected. The purpose of the positive control is to provide information on whether the algal assay is responding to a known toxic material and, after several assays have been conducted, the various positive control responses can provide data that are used to assess the variability occurring between assays. The positive control response is not used when tabulating the EC_{50} value for the final test result, but the positive control values obviously are used to help determine whether a test response is accepted as a valid test result.

The positive control contains the growth media solution, the algal cell inoculum, and the zinc chloride. It should be emphasized that the positive control compound is added directly to the assay flask and not to a soil sample. Two positive control flasks are prepared for each assay. The initial algal cell concentration should be $1 \times 10^4 \pm 1 \times 10^3$ cells/ml. The average response of the two positive controls must show at least a 15 percent inhibition as compared to the negative control. In addition, the cell counts from the two positive control flasks must be within 50 percent of each other in order to achieve a valid test result. If these conditions do not exist for the positive control result, the assay results will not constitute a valid test response. The suggested zinc chloride concentration is 128 µg/l but the suitability of this concentration should be verified by the testing laboratory prior to beginning an actual series of assays.

Several concentrations of the positive control compound should be tested prior to beginning a series of assays. This is suggested in order to verify the dose/response curve for zinc chloride (or any other compound selected for use as a positive control). Table II shows a series of zinc chloride concentrations and the resulting 96-hour algal response. Based on this series of concentrations, the EC_{50} for zinc chloride was determined to be 128.3 µg/l. As mentioned above, the positive control flasks were prepared at a zinc chloride concentration of 128 µg/l.

A series of positive control assays also were conducted to verify the expected variability between control flasks during a single assay. The results are shown in Table III and Table IV for 128 µg/l and for 74 µg/l of zinc chloride, respectively.

Based on the Table III assays, one reasonably could expect the 128 µg/l zinc chloride to produce a growth inhibiting effect of between -90.6 and -99.8 when tabulated using the given formula. In addition, the variation between the two positive control assays (%) should not be greater than 4.6. The EC_{50} determinations are not required on a routine basis (for each assay) since a series of different concentrations would be required.

TABLE I. COMPOSITION OF ALGAL GROWTH MEDIA

MACRONUTRIENTS			
Stock Solution*		Nutrient Composition Prepared Medium	
Compound	Concentration (g/l)	Element	Concentration (mg/l)
	STOCK SOLUTION I		
$NaNO_3$	25.500	N	4.200
$NaHCO_3$	15.000	Na**	11.001
		C	2.143
	STOCK SOLUTION II		
K_2HPO_4	1.044	K	0.469
		P	0.186
	STOCK SOLUTION III		
$MgSO_4$ $7H_2O$	14.700	S	1.911
	STOCK SOLUTION IV		
$MgCL_2$ $6H_2O$	12.164	Mg***	2.904
$CaCl_2$ $2H_2O$	4.410	Ca	1.202

MICRONUTRIENTS			
Stock Solution		Nutrient Composition Prepared Medium	
Compound	Concentration (mg/l)	Element	Concentration (μg/l)
	STOCK SOLUTION V		
H_3BO_3	185.520	B	32.460
$MnCl_2$ $4H_2O$	415.610	Mn	115.374
$ZnCl_2$****	3.271	Zn	1.570
$CoCl_2$ $6H_2O$****	1.428	Co	0.354
$CuCl_2$ $2H_2O$****	0.012	Cu	0.004
Na_2MoO_4 $2H_2O$****	7.260	Mo	2.878
$FeCl_3$ $6H_2O$	160.000	Fe	33.051
Na_2EDTA $2H_2O$	300.000	--	--

*Other forms of the salt may be used if the resulting element concentrations are the same as those listed.

**Includes Na from $NaNO_3$.

***Includes Mg from $MgSO_4$ $7H_2O$.

****Prepare at 100 times concentration and add in designated amount to stock solution V.

TABLE II. CHLOROPHYTA RESPONSE TO VARIOUS CONCENTRATIONS OF ZINC CHLORIDE (Positive Control Compound Added Directly to Assay Flask)

Zinc Chloride Concentration (µg/l)	Algal Cells/ml After 96 Hours	Calculated Effect Value
negative control	3.1×10^6	---
9.7	3.1×10^6	0.3
18.5	3.1×10^6	- 0.1
37.0	3.0×10^6	- 5.0
74.0	2.4×10^6	- 24.4
148.0	1.2×10^5	- 96.5
296.0	1.3×10^4	- 99.9

* Response to Positive Control = $\dfrac{(PC - IN) - G(NC - IN)}{G(NC - IN)}$

NC = cells/ml for negative control
PC = cells/ml for postivie control
G = proportion of growth media used; assumed to be 1.0 because no actual eluate is used and because only a small volume (µl) of zinc chloride solution is added
IN = cells/ml in flask immediately after addition of algal cells

** Based on this series of concentrations, the calculated EC_{50} for zinc chloride is 128.3 µg/l. The positive control compound is added directly to an assay flask and not to a soil sample.

Sample Material

Clay Loam Soil

Water eluates were taken from a series of soil types and tested using Selenastrum capricornutum before a clay loam soil (characterized as being 22% sand, 51% silt, and 27% clay) was selected for use during the evaluation. After the soil was selected, a suitable quantity (approximately 275 kg) was collected so that enough soil would be available for the entire single laboratory effort. The soil was homogenized thoroughly, first by grinding and then by mixing in a mechanical soil mixer for several hours. The soil and the subsequently prepared soil eluate were chemically analyzed primarily to determine whether a sufficiently homogenized preparation had been achieved for use in the current evaluation. Analyses also were conducted to confirm the transport of fluoride from the spiked soil (sodium fluoride) to the eluate and to compare this transport with the eventual EC_{50} test result. Results from this analytical effort are shown in Tables V, VI, and VII.

As mentioned previously, the specified assay procedure consists of soil collection, soil drying, and subsequent water addition. Consequently, for the

TABLE III. SERIES OF ALGAL ASSAYS USING 128 µg/l OF ZINC CHLORIDE (Two Separate Assays are Conducted with Positive Control Compound Added Directly to Assay Flask)

Positive Control Response

Assay Number	Calculated Effect I	Calculated Effect II	Variation Between Assays (%)
1	- 92.2	- 89.9	2.5
2	- 94.6	- 96.9	2.4
3	- 95.6	- 97.1	1.6
4	- 96.3	- 94.9	1.5
5	- 85.7	- 83.4	2.7
6	- 96.3	- 97.7	1.4
7	- 97.9	- 94.6	3.3
8	- 89.9	- 98.9	9.2
9	- 96.9	- 96.7	0.1
10	- 98.0	- 98.4	0.4
11	- 96.9	- 96.1	0.8
12	- 98.0	- 97.4	0.6
X	- 94.8	- 95.2	2.2
±SD	3.8	4.4	2.4
CV (%)	4.0	4.6	---

TABLE IV. SERIES OF ALGAL ASSAYS USING 74 µg/l OF ZINC CHLORIDE (Two Separate Assays are Conducted with Positive Control Compound Added Directly to Assay Flask)

	Positive Control Response		
Assay Number	Calculated Effect I	Calculated Effect II	Variation Between Assays (%)
1	- 45.0	- 48.3	6.9
2	- 52.0	- 50.6	1.9
3	- 53.0	- 59.3	10.7
4	- 51.0	- 69.0	26.1
5	- 54.2	- 62.2	12.9
6	- 58.8	- 50.2	14.7
$\bar{X}$ =	- 52.3	- 56.6	
±SD =	4.5	8.2	
CV (%) =	8.6	14.5	

single laboratory effort, a subsample of the homogenized test soil was taken, transferred to a ceramic pan, air dried for 24 hours, and a 125-gram portion transferred to a teflon container for water addition.

At this stage the eluate preparation process begins and 500 ml of deionized water are added to the 125 gram sample. The teflon container is capped and mechanically shaken in the dark for 48 hours at room temperature. The liquid is then decanted from the container and subsequently centrifuged. After centrifugation, the eluate is transferred to a Buchner funnel that contains a nonsterile glass fiber filter and filtered under vacuum. The eluate is subsequently filtered through a nylon filter to obtain a sterile eluate. This filtered liquid is the soil "eluate" mentioned in Tables VI and VII. It is also the material that, under routine testing conditions, is diluted with growth media and to which algal cells are added for testing.

Chemical Analysis

The chemical analyses were conducted on subsamples of the clay loam test soil and on the eluates taken from additional subsamples of the same homogenized soil collection. The only real objective was to estimate the chemical variability likely to occur (during the single laboratory evaluation) between the different soil subsamples. Variability that occurs during the soil drying phase and/or during eluate preparation will become part of the overall method error because these steps are components of this freshwater assay.

TABLE V. ELEMENT ANALYSIS OF SOIL SUBSAMPLES TAKEN FROM THE HOMOGENIZED SOIL COLLECTION USED FOR THE CHLOROPHYTA EVALUATION

Element Composition (μg/g)

Subsample Number	Mg	K	Zn	Fe	Mn	Cu	Ca	SO_4-S	pH
1	27.3	21.5	7.5	76.2	71.8	0.76	195.5	7.5	5.5
2	29.5	22.3	7.5	73.6	71.6	0.48	202.5	7.0	5.5
3	27.5	20.8	7.5	71.0	67.4	1.24	180.5	7.0	5.4
4	27.3	20.1	6.8	68.9	64.1	1.53	184.5	6.8	5.4
5	24.7	19.0	6.4	65.4	59.8	1.52	170.0	7.0	5.4
6	26.7	20.3	6.8	66.0	59.4	0.49	176.0	7.0	5.4
7	27.6	20.2	7.1	68.7	67.0	1.05	199.5	9.3	5.6
8	26.3	20.0	7.7	73.6	71.6	0.48	182.5	7.5	5.5
9	27.7	21.8	6.6	64.9	63.5	0.55	189.5	7.3	5.5
10	29.5	22.8	7.4	74.6	60.2	0.63	185.2	5.5	5.5
$\bar{X}$	27.4	20.9	7.1	70.5	65.6	0.9	185.3	7.2	5.5
±S.D.	1.4	1.2	0.5	4.4	5.0	0.4	11.3	0.9	0.1
C.V.*	5.2	5.7	6.3	6.2	7.6	49.4**	6.1	12.9	1.2

* Soil subsamples were randomly taken from the homogenized collection. Several organic and inorganic analyses have been conducted to verify the level of homogeneity achieved between subsamples. In addition to the results shown above, a few nitrogen analyses were conducted (Kjeldahl nitrogen) using the soil eluates. These analyses revealed an essentially consistent concentration (μg/ml) occurring between eluates from different soil subsamples. Some attempts at organic compound identification also were conducted, i.e., eluate samples were analyzed using GC/MS and LC/MS procedures. Few specific organics were detected and those that were identified were detected at low concentrations (ng/ml for eluates and ng/g for the actual soil). Tentatively identified organic compounds included benzoic acid, hydroxy methoxy benzaldehyde, and methyl hexanone. The various chemical analyses have helped to confirm that a reasonably homogenized soil was selected for the current Chlorophyta evaluation.

** With the exception of potential analytical error at these relatively low concentrations, no explanation is presented for this unusually high coefficient of variation.

TABLE VI. APPROXIMATE TRANSFER OF SODIUM FLUORIDE FROM SOIL TO ELUATE USING THE CHLOROPHYTA ASSAY PROCEDURE

	Sodium fluoride addition to 125-g soil sample (mg)				
	556	900	1090	1112	2224
Sodium fluoride concentration in soil (mg/g)	4.5	7.2	8.7	9.0	17.8
Sodium fluoride concentration in eluate (ug/ml)	378	442	770	693	1714
Total recovery in eluate (mg)	151.2	176.8	308.0	277.2	685.6
Approximate recovery of spike (%)	27	20	28	25	31
$\bar{X}$ EC_{50} for assay	-0.7	56.6	50.9	44.6	32.7
Standard deviation $\pm$	---	6.5	7.5	2.6	8.2
Coefficient of variation (%)	---	11.4	14.8	5.8	25.1

Several analytical methods were used. A sonication extraction procedure, which included three or more soil extractions per sample, was used for the soil analyses (EPA Method 3550). Inductively coupled argon plasma spectroscopy (ICAP) was used for the element determinations and gas chromatography-mass spectrometry, followed by a few liquid chromatography-mass spectrometry determinations, was used for the tentative identification of organic compounds. These same analytical techniques also were used for the soil eluate samples; ion chromatography and the Kjeldahl procedure were used, respectively, for fluoride and nitrogen determinations.

Replicate analyses, sample dilutions, and spiked samples were used routinely for quality control and, in some cases, standard reference samples also were available. However, reference samples were not available for the organic compounds tentatively identified at very low concentrations.

For purposes of inorganic analysis, ten soil subsamples were randomly taken from the homogenized collection. Coefficients of variation noted for the soil elements were reasonably good (Table I). With the exception of a potentially increased analytical error at low concentrations, no explanation is given for the unusually high variability noted for the copper concentrations.

TABLE VII. TRANSFER OF SOIL ELEMENTS TO THE SUBSEQUENTLY PREPARED ELUATE

	soil (μg/g)	leachate (μg/ml)	% of soil element transferred to leachate
arsenic	12.0	0.08	0.7
barium	210	2.80	1.3
chromium	11.0	0.12	1.1
cobalt	12.0	0.04	0.3
copper	8.6	0.23	2.7
lead	28.0	0.24	0.9
nickel	10.0	0.12	1.2
vanadium	46.0	0.32	0.7
zinc	55.0	0.82	1.5

Note: The soil samples that were analyzed, and the soil samples that were used to prepare eluates, were subsamples of a single soil collection. Inorganic analyses were conducted using inductively coupled argon plasma spectroscopy (ICAP). Eluates were prepared as directed by the Chlorophyta assay protocol. The transfer of soil elements to the eluate (%) is only an approximation based on an average soil concentration and an average eluate concentration. Organic material also was transferred (probably as the diversified high molecular weight organics typically grouped as humic substances) but few specific compounds were detected.

The soil apparently was well homogenized, however, and seemed to be a good material for use during the single laboratory evaluation.

The approximate transfer of fluoride from soil to eluate is shown in Table VI. While the variability shown in the table contributes to the overall method variability, it is not really necessary to characterize variations in the toxicant transfer to eluate separately from the accuracy, sensitivity, precision, etc., of the final EC_{50} result. Approximately, 400 ml of eluate is typically recovered from the original 500 ml of water added when using the homogenized clay loam test soil. When using the test soil, at least 20 to 30 percent of the sodium fluoride addition is typically recovered as fluoride in the eluate. For the original soil components, the transfer of elements from soil to water eluate is considerably less (Table VII). The transfer of soil elements to the eluate (Table VII) is only an approximation based on an average soil concentration and an average eluate concentration.

Attempts at organic compound identification, as well as some analyses for nitrogen content (i.e., Kjeldahl method where ammonia nitrogen not removed during initial phase of procedure), yielded little new information. As mentioned previously, eluate samples were analyzed using GC/MS and LC/MS procedures. Some organic material apparently was transferred from the test soil to the eluate, presumably as diversified high molecular weight compounds typically grouped as humic substances. However, few specific organics were detected during this analytical effort. Those organics that were tentatively identified (i.e., methoxybenzoic acid, hydroxy methoxy benzaldehyde, benzoic acid, etc.) were detected at low concentrations (ng/ml for eluates and ng/g for the actual soil). Nitrogen concentrations were noted at µg/g levels for some of the soil eluates.

RESULTS AND DISCUSSION

Ruggedness Test

As mentioned above, the method protocol was prepared based on an EPA-Corvallis Laboratory procedure for the bioassessment of hazardous waste sites that had previously been tested at actual waste site locations and found to be one of the better screening techniques for detecting the presence of potentially hazardous compounds. The protocol was then reviewed by several scientists familiar with the assay and was revised based on their recommendations. Many of the revisions were made to achieve completeness of protocol instruction as much as to revise individual steps of the laboratory procedure. This revised protocol was then reviewed a second time in order to ensure that a consensus method protocol had been achieved.

Although a consensus procedure is always a goal, it is not always possible to achieve complete agreement. Results of the review process approached a consensus conclusion but some differing assay approaches (primarily in the area of algal growth media composition) continued unresolved throughout the consensus process.

The "consensus procedure" was tested to confirm assay responsiveness to different types of soil samples and to different chemical compounds that were added to individual soil samples. In addition to confirming method responsiveness to different sample materials, this brief series of tests also allowed the laboratory personnel, who would be involved in the single laboratory evaluation phase to become thoroughly familiar with the revised assay procedure.

The next step in the evaluation was to identify those procedural variables that must be carefully controlled. If the Chlorophyta procedure is "rugged" it will not be susceptible to the inevitable, modest departures in routine that occur, and the final test result will not be altered by these slight variations. If the results are altered by small procedural variations, it is important to emphasize in the protocol that a specific step must be strictly followed or, in some cases, to indicate the limits of allowable variability. The experimental design used during the ruggedness test (based on the design by W. J. Youden In The Collaborative Test) did not seek to study each separate variable in an individual sequential fashion, but rather to provide for the introduction of several changes (protocol variations) simultaneously (Tables VIII and IX).

TABLE VIII. EXPERIMENTAL DESIGN FOR A SEVEN VARIABLE RUGGEDNESS TEST*

Determination Number	Combination of Variables	Test Result
1	A B C D E F G	s
2	A B c D e f g	t
3	A b C d E f g	u
4	A b c d e F G	v
5	a B C d e F g	w
6	a B c d E f G	x
7	a b C D e f G	y
8	a b c D E F g	z

*Based on W.J. Youden, 1969, The Collaborative Test, p. 151-158. In Precision Measurement and Calibration. H.H. Ku Editor. U.S. Department of Commerce, National Bureau of Standards. 436 pp. For the Chlorophyta evaluation, each test sample contained 100 mg of the organic compound sodium dodecyl sulfate that had been added to the 125-gram aliquot soil samples immediately prior to the test.

As noted in Table VIII, eight complete assays were conducted as part of the ruggedness evaluation. Each test sample contained 100 mg of the organic compound sodium dodecyl sulfate that had been added to the 125-gram aliquot soil samples immediately prior to the test. The "protocol directed" conditions were designated as A through G, and the varied conditions were designated as a through g. The evaluation was concerned with identifying respective variations in the final test result due to the specific procedural differences, i.e., A-a, B-b, C-c, D-d, E-e, F-f, and G-g. Each of the eight trials consisted of a single analysis conducted using eight aliquots of the test material (100 mg of sodium dodecyl sulfate). The final test results are indicated as s, t, u, v, w, x, y, and z.

The average of $A = (s + t + u + v)/4$, compared with the average of $a = (w + x + y + z)/4$, serves as a rapid means of assessing the effect of changing variable A to a. Because each of the two groups of four determinations contains the other six variables (twice at the upper case level and twice at the lower case level), the effect of these variables (if present) tends to cancel out, leaving only the effect of changing variable A to a. The relative effect of the other variables was estimated by examining the following averages.

TABLE IX. TEST VARIABLES USED TO EVALUATE RUGGEDNESS OF CHLOROPHYTA PROCEDURE

Directed Instruction	Altered Instruction
A. 125-g soil aliquot used as sample material for assay.	a. 110-g soil aliquot used as sample material for the assay.
B. at start of assay, each flask contains 1 x 10^4 algal cells per ml.	b. at start of assay, each flask contains 0.75 x 10^4 algal cells per ml.
C. original soil collection is first air dried for 24 hours (room temperature).	c. original soil collection is first air dried for 20 hours (room temperature).
D. 500 ml of water added to the aliquot sample of air dried soil.	d. 550 ml of water added to the aliquot sample of air dried soil.
E. soil eluate is pH adjusted to between 6.0 - 8.5 prior to the assay.	e. soil eluate is pH adjusted to pH 10 prior to the assay.
F. eluate dilution series consists of 1%, 10%, and 80% soil eluate.	f. eluate dilution series consists of 1%, 30%, and 80% soil eluate.
G. algal assay terminated at 96 hours.	g. algal assay terminated at 92 hours.

$$B = \frac{(s + t + w + x)}{4} \qquad b = \frac{(u + v + y + z)}{4}$$

$$C = \frac{(s + u + w + y)}{4} \qquad c = \frac{(t + v + x + z)}{4}$$

$$D = \frac{(s + t + y + z)}{4} \qquad d = \frac{(u + v + w + x)}{4}$$

$$E = \frac{(s + u + x + z)}{4} \qquad e = \frac{(t + v + w + y)}{4}$$

$$F = \frac{(s + v + w + z)}{4} \qquad f = \frac{(t + u + x + y)}{4}$$

$$G = \frac{(s + v + x + y)}{4} \qquad g = \frac{(t + u + w + z)}{4}$$

After tabulating the above averages, the differences between each respective variable was computed, e.g.,

$$A - a = \frac{(s + t + u + v)}{4} - \frac{(w + x + y + z)}{4}$$

Most of the modest procedural alterations should typically have little or no effect on the test result. However, a comparison of the respective differences (A-a, B-b; etc.) provides considerable information on which variables, if any, are having the greater effects.

Moderate procedural variations definitely have altered the final Chlorophyta test result when they have occurred at certain critical steps (Table X). Steps that apparently must be followed strictly as written (variables D, E, and F) concern the amount of water added to the dry soil sample, the pH of the resulting soil eluate that is actually used during the assay, and the specific eluate concentrations (dilutions) prepared for the assay dilution series. The Chlorophyta protocol consequently was revised to emphasize strict adherence to these critical instructions.

Precision

In order to interpret the significance of any test result, the method's intrinsic capability for precision must be known. Precision is basically a measure of repeatability or the amount of variability resulting when replicate samples are tested at either approximately the same time or at different times. There are several approaches to estimating precision, all of which provide information addressing the confidence a laboratory can have in the repeatability or reproducibility of a given assay result. A single laboratory test, such as this Chlorophyta method evaluation, is concerned with a measure of within-laboratory (random or indeterminate) error. However, a single laboratory evaluation does not provide information on between-laboratory error which can only be determined through a multilaboratory evaluation. The multilaboratory evaluation (collaborative test) obviously is necessary if a method gains wide application and several laboratories are using a given procedure to provide monitoring information.

The precision evaluation was conducted using the ruggedness tested, and ruggedness revised, Chlorophyta method protocol. Each sample contained 8.9 mg of sodium fluoride per gram of homogenized clay loam soil. This sodium fluoride concentration was selected, somewhat in retrospect after several concentrations were tested, because the assay response was close to the calculated EC_{50} concentration and because the final result seems to represent the best attainable precision when using the Chlorophyta procedure.

Ten separate determinations using aliquots of the same sample were conducted. Each separate determination shown in Table XI represents a valid test result as required by the Chlorophyta method protocol. Based on the 10

TABLE X. RELATIVE EFFECT OF CHLOROPHYTA PROTOCOL VARIATIONS ON FINAL TEST RESULT

Test Results Using Different Combinations of Protocol Variables*

Determination	Test Result
s	30.5
t	79.1
u	29.9
v	43.4
w	21.8
x	45.1
y	90.8
z	47.9

Relative Effect of Individual Variations**

A - a =	- 5.7	E - e =	- 20.4
B - b =	- 8.9	F - f =	- 25.3
C - c =	- 10.6	G - g =	7.8
D - d =	27.0		

* Calculated soil eluate dilution that will result in an EC_{50} value using Selenastrum capricornutum. Letters s through z represent different variable combinations (Table VIII). Each test sample contained 100 mg of the organic compound sodium dodecyl sulfate, which had been added to the 125-gram aliquot soil samples immediately prior to the test.

**The protocol directed conditions are designated as A through G, and the slightly altered conditions are designated as a through g (Table IX).

separate determinations using 8.9 mg sodium fluoride per gram of soil, the assay's single laboratory capability for precision (expressed as a coefficient of variation) is presented as 5.8 percent. An attempt was made to conduct the individual determinations on alternate days, i.e., an interval of at least one day between the completion of one assay and the start of the next. While an exactly identical interval was not maintained between individual assays due to the eluate preparation time and problems of routine laboratory scheduling, the individual analyses shown in Table XI were conducted sequentially over a total time interval that covered several days.

Response to Organic Compound

Soil containing 2,4-dichlorophenol also was tested using the Chlorophyta procedure. Chlorinated hydrocarbons frequently are present at hazardous waste site locations and they definitely represent one of the waste site chemical

TABLE XI. CHLOROPHYTA ASSAY'S SINGLE LABORATORY CAPABILITY FOR PRECISION (Sample Material Consisted of 8.9 mg Sodium Fluoride per Gram of Soil)

Assay Number	Calculated EC_{50}	Positive Control			Negative Control	Valid Test Result Achieved
		#1	#2	% Difference		
1	41.0	-53.0	-59.3	8.9	2.9×10^6/ml	yes
2	47.4	-51.0	-69.0	26.1	2.8×10^6/ml	yes
3	41.1	-51.0	-59.5	14.3	2.7×10^6/ml	yes
4	42.1	-58.8	-50.2	14.7	2.6×10^6/ml	yes
5	46.6	-62.9	-72.9	13.9	2.6×10^6/ml	yes
6	44.8	-57.7	-65.7	12.2	2.6×10^6/ml	yes
7	46.0	-72.0	-80.4	10.0	2.7×10^6/ml	yes
8	43.5	-89.5	-91.1	2.0	2.6×10^6/ml	yes
9	45.5	-93.6	-94.8	1.3	2.5×10^6/ml	yes
10	48.2	-72.0	-76.1	5.4	2.5×10^6/ml	yes
$\bar{X}$	44.6					
±S.D.	2.6					
C.V.(%)	5.8					

* Based on 10 separate determinations using 8.9 mg sodium fluoride per gram of soil, the assay's single laboratory capability for precision (expressed as a coefficient of variation) is presented as 5.8 percent.

** Each individual soil sample weighed 125 grams and 1112 mg of sodium fluoride was added to each sample.

*** As directed by the method protocol, the assay result was not considered to be a valid test result unless (1) the negative control had achieved at least 1.0×10^6 cells/ml at the 96-hour time interval, (2) an average of the two positive control results demonstrated at least a 15 percent inhibition in cell growth, and (3) the two positive control results did not differ from each other by more than 50 percent.

groups that cause environmental concern. This particular chemical was tested as a representative organic pollutant that is somewhat resistant to microbial degradation.

The dichlorophenol was added to the clay loam test soil to achieve a concentration of 80 µg/g. Separate determinations were conducted using ten identical soil samples that contained this concentration of dichlorophenol. Each of the ten determinations represented a valid test response as directed by the Chlorophyta method protocol. It has already been established that the Chlorophyta procedure was responsive to organic pollutants as well as to actual waste site samples that contain low concentrations of several chemicals. This method responsiveness was certainly present when the soil samples contained dichlorophenol. As is noted in Table XII, however, the observed precision is not very impressive even as compared to the test results achieved using sodium fluoride. Since preliminary testing preceded selection of the 80 µg/g dichlorophenol concentration, these data may suggest a responsive procedure but a somewhat less precise assay than expected when the sample contains an organic pollutant (i.e., 46.5 percent coefficient of variation when testing dichlorophenol).

Method Sensitivity

For purposes of a single laboratory evaluation, a method's sensitivity is defined as the method's capability to detect (or distinguish between) small changes in sample concentration, i.e., concentrations of analyte. The specific concentrations for the sensitivity phase usually are selected sequentially based on results from previously selected concentrations. The same sample material (sodium fluoride in clay loam soil) was used for the method sensitivity evaluation as was used during the precision test phase. Table XIII presents a series of assay results that were ultimately used to assess the method's capability not only for sensitivity but also for the limits of reliable measurement.

The single laboratory evaluation typically uses ten independent assays for each new concentration (i.e., ten separate valid responses acquired by following the written protocol) and this was done during the current effort. Under routine operating conditions, however, the assay would only be conducted one time per sample, and, consequently, when tabulating data for sensitivity, non-overlapping standard deviations (rather than non-overlapping standard errors) are used to indicate whether the method can distinguish between the different samples.

As noted in Table XIII, the Chlorophyta procedure does not have a particularly impressive capability for sensitivity. Concentrations of sodium fluoride between 17.8 mg/g and 48.0 mg/g of soil produced the same test response. In addition, concentrations between 8.9 mg/g and 7.2 mg/g of soil produced the same test response. Unfortunately, this total concentration range also covers most of the response range. The Chlorophyta procedure, therefore, clearly will detect the presence of the pollutant compound but, for the most part, is only sensitive to order of magnitude concentration differences.

Limits of Reliable Measurement

The same sample material should be used for reliability measurement as was originally used during the method precision and method sensitivity evaluations.

TABLE XII. ASSAY RESPONSE USING SOIL SAMPLES CONTAINING EITHER SODIUM FLUORIDE OR 2,4-DICHLOROPHENOL (Precision Capability, Expressed as a Coefficient of Variation, in Response to Soil Containing Either an Inorganic or an Organic Pollutant Compound)

	Final Result Expressed as Calculated EC_{50}	
Assay Number	1112 mg NaF per 125 g soil (8.9 mg/g)	10 mg dichlorophenol per 125 g soil (80 µg/g)
1	41.0	55.9
2	47.4	57.3
3	41.1	49.8
4	42.1	25.7
5	46.6	93.4
6	44.8	40.2
7	46.0	94.7
8	43.5	46.1
9	45.5	44.5
10	48.2	116.2
$\bar{X}$ EC_{50}	44.6	62.4
± SD	2.6	29.0
CV(%)	5.8	46.5

The evaluating laboratory typically should select two concentrations of sample material. One of these concentrations should be near the upper extreme of the method's detection range and the other concentration should be near the lower extreme of the detection range. Ten analyses would be conducted on each concentration to provide precision data (expressed as a coefficient of variation). However, the previously acquired data confirming the poor capability for sensitivity and the somewhat limited total assay response range made this stage of the evaluation less difficult than is usually the case.

As was the case for the above-mentioned method sensitivity, the limits of reliable measurement are not particularly impressive other than that the assay does show a definitive response to the pollutant over a reasonable concentration range (6.4 mg/g to 48.0 mg/g before complete inhibition/lethality). For this phase, the basic objective was to verify that the method capabilities for sensitivity, precision, and accuracy (when applicable) do not deteriorate at the upper and lower extremes of the detection range.

Although the procedure's sensitivity is not very good at the extremes of the detection range, it is not any less impressive than the method's best sensitivity. The method's deterioration in precision provides the most information concerning limits of reliable measurement. As mentioned above, ten analyses were conducted at each of several concentrations (Table XIII) so that, among other reasons, precision comparisons could be made. The coefficient of variation frequently will show a dramatic increase at the extreme limits of detection and, therefore, precision data provide a distinct indication of the limits of reliable measurement. The results of this effort suggest a very

TABLE XIII. RESPONSE OF CHLOROPHYTA ASSAY PROCEDURE TO VARIOUS SOIL CONCENTRATIONS OF SODIUM FLUORIDE

mg sodium fluoride per 125 g of soil	mg sodium fluoride per gram of clay loam soil	Mean EC_{50}	± SD	CV (%)	Range of EC_{50} ± SD
556	4.5	-0.7	--	--	--
700	5.6	-181.7	455.3	250.6	--
800	6.4	54.3	6.9	12.7	47.4 - 61.2
900	7.2	56.6	6.5	11.4	50.1 - 63.1
1090	8.7	50.9	7.5	14.8	43.4 - 58.4
1112	8.9	44.6	2.6	5.8	42.0 - 47.2
2224	17.8	32.7	8.2	25.1	24.5 - 40.9
3336	26.7	31.9	9.1	28.5	22.8 - 41.0
4448	35.6	22.6	8.1	35.8	14.5 - 30.7
6000	48.0	16.6	10.9	65.5	5.7 - 27.5
7500	60.0	-0.9	29.5	--	--

Note: Each mean EC_{50} valve is the result of ten separate assays using the indicated concentration of sodium fluoride per gram of soil. Each separate assay was conducted as directed by the method protocol and included the required control samples. Concentrations of 4.5, 5.6, and 60.0 mg/g apparently are outside the limits of reliable measurement for the Chlorophyta assay. Resulting algal cell concentrations for the specific assays are given in Appendix A.

restricted concentration range of 6.4 mg/g to 35.6 mg/g of soil. The coefficient of variation at the greater concentrations increases rapidly, especially when compared with the 5.8 percent coefficient of variation achieved at the 8.9 mg/g concentration. The 65.5 percent noted at 48.0 mg/g might well be considered as being beyond a range of reliable measurement. However, the assay clearly shows a response to sodium fluoride at the increased concentrations and, considering that the overall procedure is only a screening technique, the increased coefficient of variation should not really affect method useability.

Accuracy

To determine a method's single laboratory capability for accuracy (and for

systematic error), the testing laboratory must have both a standard reference material and a known method response (true response) to this reference material. When a method calls for analysis of biological tissue or biological fluid, there will usually be a standard reference material available to the testing laboratory, e.g., samples with certified compound concentrations or perhaps with certified enzymatic activity levels. In these instances, the method's single laboratory capability for accuracy can be assessed by determining the differences between the observed single laboratory result, using the reference sample, and the known true value.

In the case of a method such as this algal assay that uses the soil eluates, no "true response" is available for a reference material; hence the method's single laboratory capability for accuracy (or for systematic error) cannot be determined. Under these conditions, the testing laboratory should first select a reference material and then determine an average test response for a single protocol. Comparison of test data with the results of a reference method can be used in certain situations, but for the purposes of this evaluation, the use of a reference method is not really possible and is not typically done for a single laboratory evaluation.

Although not technically a standard, this testing phase used a white silica sand that is recommended by the American Society for Testing and Materials (ASTM) for use in certain cement preparations and, more significantly, can be acquired easily by other laboratories that are using the assay procedure. The sand has a particle size of 850 to 600 μm and receives a series of agitation washes prior to use. It is subsequently spiked with sodium fluoride to achieve a 4.8 mg/g concentration.

A greater recovery of toxic chemical will typically occur in the eluate when sand is used and, consequently, the known test response is being established at a different sodium fluoride concentration than would be the case with the clay loam soil that was used as sample material during most of the current evaluation. The known test response (Freshwater Assay Using Soil as Sample Material) to 4.8 mg of sodium fluoride per gram of washed ASTM silica sand is 43.4 ± 9.7 when expressed as the calculated $EC_{50} \pm$ the standard error. Results from the individual determinations that have provided this known value are shown in Table XIV. However, these data again emphasize an apparent poor capability for precision which, in some ways, probably detracts from the future application of this response for use in accuracy and/or systematic error estimates.

CONCLUSION

The current effort has attempted to establish the data quality that can be achieved when using the algal assay (Freshwater Assay Using Soil Eluates as Sample Material). This effort began with a "consensus" method protocol and, from that stage on, all subsequent assays strictly followed the written method procedure. The ruggedness test phase demonstrated that certain moderate procedural variations definitely altered the final Chlorophyta test result when the variations were introduced at certain critical steps. Although the procedure was not particularly rugged, the method protocol was revised to emphasize strict adherence to the more critical instructions. The complete method protocol is included in Appendix B.

TABLE XIV. DETERMINATIONS TO ESTABLISH A KNOWN TEST RESPONSE TO A REFERENCE SAMPLE (Each Final Result Expressed as Calculated EC_{50})

Assay Number	600 mg NaF per 125 g ASTM silica sand (4.8 mg/g)
1	48.2
2	48.5
3	52.5
4	62.0
5	43.5
6	29.8
7	15.1
8	25.4
9	62.2
10	21.9
11	54.2
12	23.2
13	39.8
14	45.5
15	62.1
16	50.3
17	45.3
18	43.5
19	42.6
20	51.6

$\bar{X}$ EC_{50} = 43.4
± SE = 9.7
± SD = 13.8
CV % = 31.8

These step-by-step Appendix B instructions approach being a consensus procedure (as best as could be achieved) and they include the revisions resulting from the ruggedness test phase of the current evaluation. The assay is "final" only in so far as it is (1) the procedure that was used for the single laboratory evaluation and (2) is the procedure that will be considered for collaborative testing.

Based on 10 separate determinations using 8.9 mg of sodium fluoride per gram of soil, the assay's single laboratory capability for precision (expressed as a coefficient of variation) has been presented as 5.8 percent. The procedure thus demonstrated a good capability for precision; however, the coefficient of variation increased rapidly at the more extreme concentrations revealing that the Chlorophyta assay did not have a particularly impressive capability for sensitivity and that the assay had a somewhat limited range of reliable measurement (6.4 mg/g to 35.6 mg/g of soil).

Soil containing 2,4-dichlorophenol also was tested during the current evaluation and several tests were conducted to confirm the dose/response curve using the positive control compound zinc chloride. A known test response also was established using a white silica sand spiked with sodium fluoride.

Although considerable progress has been made with the standardization of this procedure, some difficulties remain if one considers a continuation to collaborative testing. The known test response provides a value with a fairly great standard error that, in some ways, probably detracts from the future application of this response for use in accuracy and/or systematic error estimates. Another difficulty might be the fact that complete agreement was never totally reached on the original method protocol (consensus procedure). In addition, the poor capability for sensitivity and the somewhat limited range of reliable measurement might also be considered as being discouraging. However, the procedure definitely will detect the presence of pollutant compounds and, by design, the procedure is used as a screening technique. Consequently, the observed method sensitivity (ability to distinguish between different concentrations of the same material) should not be considered as being detrimental to further method standardization or to future application as part of a monitoring network. The greatest problem confronting a continuation to collaborative testing may be the recurring lack of consensus agreement noted among knowledgeable biologists for several phases of the method protocol. However, many frequently used procedures have never enjoyed total consensus agreement and various organizations such as the Association of Official Analytical Chemists (AOAC) and the American Society for Testing and Materials (ASTM) frequently publish method revisions as well as interim protocols to allow for ongoing improvements.

The authors recommend that the current assay be collaboratively tested as a next step toward more frequent application of this algal procedure for hazardous waste monitoring.

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APPENDIX A

ALGAL CELL CONCENTRATIONS

APPENDIX TABLE I. ALGAL CELL CONCENTRATION DETERMINED AT 96-HOUR INTERVAL IN RESPONSE TO ELUATE FROM 80.0 µg/g DICHLOROPHENOL SOIL SAMPLE (All Values, Including 96-Hour Response for Negative and Positive Controls, Given as Cell Number/ml)

Assay Number	Growth Media	Eluate Dilution 1%	Eluate Dilution 10%	Eluate Dilution 80%	Positive Control #1	Positive Control #2
1	3.1×10^6	2.4×10^6	1.7×10^6	2.6×10^5	2.4×10^6	2.4×10^6
2	2.4×10^6	2.1×10^6	1.4×10^6	1.9×10^5	8.2×10^5	7.1×10^5
3	2.6×10^6	2.6×10^6	1.3×10^6	1.9×10^5	2.1×10^6	1.9×10^6
4	2.9×10^6	2.6×10^6	7.8×10^5	1.4×10^5	1.9×10^6	2.0×10^6
5	2.8×10^6	3.1×10^6	2.5×10^6	3.4×10^5	9.9×10^5	1.0×10^6
6	2.5×10^6	2.4×10^6	2.0×10^6	2.7×10^4	1.2×10^6	9.7×10^5
7	3.0×10^6	3.5×10^6	2.5×10^6	3.7×10^5	2.2×10^6	2.3×10^6
8	2.4×10^6	2.9×10^6	7.9×10^5	1.4×10^5	1.5×10^6	1.3×10^6
9	3.2×10^6	3.1×10^6	2.7×10^6	5.8×10^4	2.6×10^6	2.5×10^6
10	3.6×10^6	3.5×10^6	2.2×10^6	4.6×10^5	1.4×10^6	1.4×10^6

Note: The sample material consisted of clay loam soil (characterized as being 22% sand, 51% silt, and 27% clay) that contained dichlorophenol at the noted concentration. The positive control contained the growth medium solution, the algal cell inoculum, and zinc chloride (128 µg/l). The negative control was prepared by adding the algal cells to an undiluted aliquot of growth medium. At the end of a 96-hour test, the negative control sample should range from 1.6×10^6 to 2.6×10^6 cells/ml.

APPENDIX TABLE II. ALGAL CELL CONCENTRATION DETERMINED AT 96-HOUR INTERVAL IN RESPONSE TO ELUATE FROM 4.5 mg/g SODIUM FLUORIDE SOIL SAMPLE (All Values, Including 96-Hour Response for Negative and Positive Controls, Given as Cell Number/ml)

Assay Number	Growth Media	Eluate Dilution 1%	Eluate Dilution 10%	Eluate Dilution 80%	Positive Control #1	Positive Control #2
1	2.6×10^6	2.0×10^6	2.5×10^6	1.7×10^6	1.0×10^6	1.1×10^6
2	1.9×10^6	1.1×10^6	1.7×10^6	1.5×10^6	6.3×10^5	5.9×10^5
3	2.7×10^6	1.6×10^6	2.4×10^6	2.1×10^6	1.8×10^5	3.0×10^5
4	2.5×10^6	1.0×10^6	1.6×10^6	1.8×10^6	6.4×10^5	4.3×10^5
5	1.6×10^6	5.4×10^5	1.8×10^6	8.0×10^5	1.7×10^5	1.8×10^5
6	2.8×10^6	9.5×10^5	2.6×10^6	2.2×10^6	6.0×10^4	1.2×10^5
7	1.1×10^6	3.6×10^5	1.0×10^6	7.1×10^5	6.9×10^5	5.0×10^5
8	2.8×10^6	7.1×10^5	2.2×10^6	1.3×10^6	1.1×10^5	2.0×10^5
9	1.9×10^6	2.6×10^5	8.6×10^5	6.7×10^5	9.7×10^5	9.0×10^5
10	2.2×10^6	2.0×10^6	2.2×10^6	1.4×10^6	1.6×10^6	1.6×10^6

Note: The sample material consisted of clay loam soil (characterized as being 22% sand, 51% silt, and 27% clay) that contained sodium fluoride at the noted concentration. The positive control contained the growth medium solution, the algal cell inoculum, and zinc chloride (128 µg/l). The negative control was prepared by adding the algal cells to an undiluted aliquot of growth medium. At the end of a 96-hour test, the negative control sample should range from 1.6×10^6 to 2.6×10^6 cells/ml.

APPENDIX TABLE III. ALGAL CELL CONCENTRATION DETERMINED AT 96-HOUR INTERVAL IN RESPONSE TO ELUATE FROM 5.6 mg/g SODIUM FLUORIDE SOIL SAMPLES (All Values, Including 96-Hour Response for Negative and Positive Controls, Given as Cell Number/ml)

Assay Number	Growth Media	Eluate Dilution 1%	Eluate Dilution 10%	Eluate Dilution 80%	Positive Control #1	Positive Control #2
1	2.4×10^6	2.3×10^6	3.1×10^6	5.2×10^5	2.9×10^5	1.8×10^5
2	2.3×10^6	1.5×10^6	1.7×10^6	2.9×10^5	8.2×10^4	6.0×10^4
3	1.6×10^6	2.5×10^6	2.8×10^6	3.8×10^5	1.4×10^5	1.4×10^5
4	2.5×10^6	2.8×10^6	2.6×10^6	8.5×10^5	5.0×10^5	4.2×10^5
5	1.6×10^6	1.7×10^6	2.1×10^6	4.0×10^5	5.1×10^5	4.1×10^5
6	2.0×10^6	2.2×10^6	3.0×10^6	7.0×10^5	5.1×10^5	6.1×10^5
7	1.6×10^6	1.0×10^6	2.1×10^6	4.8×10^5	1.3×10^6	1.3×10^6
8	1.6×10^6	1.6×10^6	2.3×10^6	3.6×10^5	9.6×10^5	1.1×10^6
9	1.0×10^6	1.2×10^6	1.5×10^6	5.5×10^5	5.8×10^5	5.4×10^5
10	1.8×10^6	9.6×10^5	1.8×10^6	7.9×10^5	1.2×10^6	1.0×10^6

Note: The sample material consisted of clay loam soil (characterized as being 22% sand, 51% silt, and 27% clay) that contained sodium fluoride at the noted concentration. The positive control contained the growth medium solution, the algal cell inoculum, and zinc chloride (128 µg/l). The negative control was prepared by adding the algal cells to an undiluted aliquot of growth medium. At the end of a 96-hour test, the negative control sample should range from 1.6×10^6 to 2.6×10^6 cells/ml.

APPENDIX TABLE IV. ALGAL CELL CONCENTRATION DETERMINED AT 96-HOUR INTERVAL IN RESPONSE TO ELUATE FROM 6.4 mg/g SODIUM FLUORIDE SOIL SAMPLES (All Values, Including 96-Hour Response for Negative and Positive Controls, Given as Cell Number/ml)

Assay Number	Growth Media	Eluate Dilution 1%	Eluate Dilution 10%	Eluate Dilution 80%	Positive Control #1	Positive Control #2
1	2.5×10^6	2.0×10^6	2.3×10^6	1.0×10^5	2.0×10^5	2.6×10^5
2	3.2×10^6	2.9×10^6	3.1×10^6	8.7×10^4	2.9×10^5	3.5×10^5
3	2.7×10^6	2.7×10^6	3.1×10^6	1.9×10^5	1.3×10^5	1.4×10^5
4	3.4×10^6	2.9×10^6	3.1×10^6	1.1×10^5	1.1×10^6	1.0×10^6
5	2.6×10^6	2.5×10^6	3.0×10^6	6.4×10^4	1.4×10^6	1.4×10^6
6	2.1×10^6	1.6×10^6	2.3×10^6	9.6×10^4	3.5×10^4	3.0×10^4
7	2.5×10^6	3.1×10^6	3.3×10^6	7.1×10^4	2.1×10^4	2.4×10^4
8	2.0×10^6	1.9×10^6	2.6×10^6	9.6×10^4	1.2×10^6	1.1×10^6
9	3.0×10^6	2.6×10^6	2.7×10^6	1.9×10^5	1.7×10^6	1.9×10^6
10	2.9×10^6	2.0×10^6	2.7×10^6	1.6×10^5	1.9×10^6	1.8×10^6

Note: The sample material consisted of clay loam soil (characterized as being 22% sand, 51% silt, and 27% clay) that contained sodium fluoride at the noted concentration. The positive control contained the growth medium solution, the algal cell inoculum, and zinc chloride (128 µg/l). The negative control was prepared by adding the algal cells to an undiluted aliquot of growth medium. At the end of a 96-hour test, the negative control sample should range from 1.6×10^6 to 2.6×10^6 cells/ml.

APPENDIX TABLE V. ALGAL CELL CONCENTRATION DETERMINED AT 96-HOUR INTERVAL IN RESPONSE TO ELUATE FROM 7.2 mg/g SODIUM FLUORIDE SOIL SAMPLES (All Values, Including 96-Hour Response for Negative and Positive Controls, Given as Cell Number/ml)

Assay Number	Growth Media	Eluate Dilution 1%	Eluate Dilution 10%	Eluate Dilution 80%	Positive Control #1	Positive Control #2
1	2.3×10^6	2.8×10^6	3.5×10^6	1.3×10^5	5.4×10^5	1.1×10^6
2	2.5×10^6	1.6×10^6	3.2×10^6	8.9×10^4	6.8×10^5	7.1×10^5
3	2.7×10^6	2.4×10^6	3.2×10^6	9.0×10^4	3.2×10^5	5.8×10^5
4	3.0×10^6	3.1×10^6	2.8×10^6	9.1×10^4	3.7×10^5	5.4×10^5
5	3.3×10^6	2.7×10^6	2.8×10^6	2.0×10^5	1.7×10^6	1.7×10^6
6	1.7×10^6	2.5×10^6	2.3×10^6	4.2×10^4	1.3×10^6	1.3×10^6
7	3.0×10^6	2.6×10^6	2.7×10^6	2.5×10^5	9.9×10^5	7.2×10^5
8	3.0×10^6	3.2×10^6	3.2×10^6	7.1×10^4	1.7×10^6	1.6×10^6
9	2.8×10^6	2.5×10^6	2.9×10^6	9.4×10^4	1.7×10^6	1.9×10^6
10	2.0×10^6	2.0×10^6	2.3×10^6	7.2×10^4	5.9×10^5	6.3×10^5

Note: The sample material consisted of clay loam soil (characterized as being 22% sand, 51% silt, and 27% clay) that contained sodium fluoride at the noted concentration. The positive control contained the growth medium solution, the algal cell inoculum, and zinc chloride (128 µg/l). The negative control was prepared by adding the algal cells to an undiluted aliquot of growth medium. At the end of a 96-hour test, the negative control sample should range from 1.6×10^6 to 2.6×10^6 cells/ml.

APPENDIX TABLE VI. ALGAL CELL CONCENTRATION DETERMINED AT 96-HOUR INTERVAL IN RESPONSE TO ELUATE FROM 8.7 mg/g SODIUM FLUORIDE SOIL SAMPLES (All Values, Including 96-Hour Response for Negative and Positive Controls, Given as Cell Number/ml)

Assay Number	Growth Media	Eluate Dilution 1%	Eluate Dilution 10%	Eluate Dilution 80%	Positive Control #1	Positive Control #2
1	2.5×10^6	2.9×10^6	2.5×10^6	5.7×10^4	2.2×10^6	2.0×10^6
2	2.7×10^6	2.6×10^6	2.2×10^6	5.4×10^4	1.6×10^6	1.4×10^6
3	2.2×10^6	2.6×10^6	2.2×10^6	5.5×10^4	1.5×10^6	1.6×10^6
4	3.0×10^6	2.9×10^6	2.6×10^6	9.3×10^4	1.4×10^6	1.4×10^6
5	2.6×10^6	3.1×10^6	2.6×10^6	1.8×10^5	1.7×10^6	1.6×10^6
6	3.3×10^6	3.2×10^6	2.2×10^6	7.2×10^4	1.6×10^6	1.6×10^6
7	2.8×10^6	2.7×10^6	2.6×10^6	7.9×10^4	1.6×10^6	1.7×10^6
8	2.6×10^6	2.6×10^6	2.9×10^6	1.3×10^5	2.0×10^6	2.1×10^6
9	2.4×10^6	3.2×10^6	3.2×10^6	6.1×10^4	1.7×10^6	1.8×10^6
10	2.3×10^6	2.4×10^6	2.0×10^6	5.0×10^4	1.4×10^6	1.3×10^6

Note: The sample material consisted of clay loam soil (characterized as being 22% sand, 51% silt, and 27% clay) that contained sodium fluoride at the noted concentration. The positive control contained the growth medium solution, the algal cell inoculum, and zinc chloride (128 µg/l). The negative control was prepared by adding the algal cells to an undiluted aliquot of growth medium. At the end of a 96-hour test, the negative control sample should range from 1.6×10^6 to 2.6×10^6 cells/ml.

APPENDIX TABLE VII. ALGAL CELL CONCENTRATION DETERMINED AT 96-HOUR INTERVAL IN RESPONSE TO ELUATE FROM 8.9 mg/g SODIUM FLUORIDE SOIL SAMPLES (All Values, Including 96-Hour Response for Negative and Positive Controls, Given as Cell Number/ml)

Assay Number	Growth Media	Eluate Dilution 1%	Eluate Dilution 10%	Eluate Dilution 80%	Positive Control #1	Positive Control #2
1	2.9×10^6	2.8×10^6	2.2×10^6	5.0×10^5	1.4×10^6	1.2×10^6
2	2.8×10^6	2.8×10^6	2.2×10^6	1.0×10^6	1.4×10^6	8.9×10^5
3	2.7×10^6	2.6×10^6	2.0×10^6	4.7×10^5	1.2×10^6	1.0×10^6
4	2.6×10^6	2.6×10^6	1.9×10^6	4.0×10^5	1.1×10^6	1.3×10^6
5	2.6×10^6	2.9×10^6	2.1×10^6	5.5×10^5	9.7×10^5	7.1×10^5
6	2.6×10^6	2.4×10^6	2.3×10^6	9.0×10^5	1.2×10^6	9.6×10^5
7	2.7×10^6	2.8×10^6	2.3×10^6	5.7×10^5	7.8×10^5	5.4×10^5
8	2.6×10^6	2.5×10^6	2.1×10^6	5.7×10^5	2.8×10^5	2.4×10^5
9	2.5×10^6	2.5×10^6	2.2×10^6	5.2×10^5	1.7×10^5	1.4×10^5
10	2.5×10^6	2.3×10^6	2.4×10^6	6.8×10^4	7.0×10^5	6.0×10^5

Note: The sample material consisted of clay loam soil (characterized as being 22% sand, 51% silt, and 27% clay) that contained sodium fluoride at the noted concentration. The positive control contained the growth medium solution, the algal cell inoculum, and zinc chloride (128 µg/l). The negative control was prepared by adding the algal cells to an undiluted aliquot of growth medium. At the end of a 96-hour test, the negative control sample should range from 1.6×10^6 to 2.6×10^6 cells/ml.

APPENDIX TABLE VIII. ALGAL CELL CONCENTRATION DETERMINED AT 96-HOUR INTERVAL IN RESPONSE TO ELUATE FROM 17.8 mg/g SODIUM FLUORIDE SOIL SAMPLES (All Values, Including 96-Hour Response for Negative and Positive Controls, Given as Cell Number/ml)

Assay Number	Growth Media	Eluate Dilution 1%	Eluate Dilution 10%	Eluate Dilution 80%	Positive Control #1	Positive Control #2
1	3.5×10^6	2.8×10^6	2.7×10^6	1.5×10^5	1.6×10^6	1.6×10^6
2	2.3×10^6	2.5×10^6	7.6×10^5	2.4×10^4	1.9×10^5	2.4×10^5
3	2.4×10^6	2.4×10^6	2.3×10^6	9.3×10^4	1.4×10^5	8.5×10^4
4	2.4×10^6	2.3×10^6	7.6×10^5	2.5×10^4	1.2×10^6	7.9×10^4
5	2.2×10^6	2.6×10^6	8.9×10^5	2.4×10^4	8.9×10^4	1.2×10^5
6	2.4×10^6	2.4×10^6	8.2×10^5	2.3×10^4	1.1×10^5	6.7×10^4
7	2.9×10^6	2.5×10^6	1.5×10^6	1.9×10^4	7.2×10^4	1.6×10^5
8	2.8×10^6	2.1×10^6	1.8×10^6	2.6×10^4	3.0×10^5	4.1×10^5
9	2.6×10^6	2.2×10^6	1.2×10^6	2.3×10^4	9.2×10^4	9.5×10^4
10	2.8×10^6	2.9×10^6	1.5×10^6	2.4×10^4	6.6×10^4	5.4×10^4

Note: The sample material consisted of clay loam soil (characterized as being 22% sand, 51% silt, and 27% clay) that contained sodium fluoride at the noted concentration. The positive control contained the growth medium solution, the algal cell inoculum, and zinc chloride (128 µg/l). The negative control was prepared by adding the algal cells to an undiluted aliquot of growth medium. At the end of a 96-hour test, the negative control sample should range from 1.6×10^6 to 2.6×10^6 cells/ml.

APPENDIX TABLE IX. ALGAL CELL CONCENTRATION DETERMINED AT 96-HOUR INTERVAL IN RESPONSE TO ELUATE FROM 26.7 mg/g SODIUM FLUORIDE SOIL SAMPLES (All Values, Including 96-Hour Response for Negative and Positive Controls, Given as Cell Number/ml)

Assay Number	Growth Media	Eluate Dilution 1%	Eluate Dilution 10%	Eluate Dilution 80%	Positive Control #1	Positive Control #2
1	2.5×10^6	2.8×10^6	7.9×10^5	2.2×10^4	1.7×10^6	1.9×10^6
2	2.5×10^6	2.6×10^6	1.3×10^6	1.9×10^4	2.0×10^6	2.0×10^6
3	2.6×10^6	2.8×10^6	1.0×10^6	2.3×10^4	1.6×10^6	1.9×10^6
4	1.8×10^6	2.5×10^6	6.9×10^5	1.8×10^4	1.2×10^6	1.1×10^6
5	2.9×10^6	2.9×10^6	8.8×10^5	1.4×10^4	2.1×10^6	2.0×10^6
6	2.1×10^6	2.4×10^6	9.8×10^5	1.5×10^4	1.1×10^6	1.6×10^6
7	1.6×10^6	2.8×10^6	9.7×10^5	1.9×10^4	3.7×10^5	4.9×10^5
8	1.8×10^6	2.0×10^6	5.6×10^5	1.4×10^4	1.9×10^5	2.3×10^5
9	2.8×10^6	2.5×10^6	1.4×10^6	2.0×10^4	1.3×10^6	1.3×10^6
10	2.5×10^6	2.0×10^6	6.5×10^5	1.6×10^4	3.8×10^5	3.3×10^5

Note: The sample material consisted of clay loam soil (characterized as being 22% sand, 51% silt, and 27% clay) that contained sodium fluoride at the noted concentration. The positive control contained the growth medium solution, the algal cell inoculum, and zinc chloride (128 µg/l). The negative control was prepared by adding the algal cells to an undiluted aliquot of growth medium. At the end of a 96-hour test, the negative control sample should range from 1.6×10^6 to 2.6×10^6 cells/ml.

APPENDIX TABLE X. ALGAL CELL CONCENTRATION DETERMINED AT 96-HOUR INTERVAL IN RESPONSE TO ELUATE FROM 35.6 mg/g SODIUM FLUORIDE SOIL SAMPLES (All Values, Including 96-Hour Response for Negative and Positive Controls, Given as Cell Number/ml)

Assay Number	Growth Media	Eluate Dilution 1%	Eluate Dilution 10%	Eluate Dilution 80%	Positive Control #1	Positive Control #2
1	2.3×10^6	2.3×10^6	1.2×10^6	2.2×10^4	2.3×10^5	3.3×10^5
2	2.4×10^6	2.1×10^6	6.7×10^5	2.0×10^4	9.5×10^4	1.2×10^5
3	2.9×10^6	1.8×10^6	1.3×10^6	2.0×10^4	2.7×10^5	2.3×10^5
4	3.0×10^6	2.6×10^6	1.1×10^6	1.4×10^4	8.9×10^5	7.0×10^5
5	1.6×10^6	1.1×10^6	1.5×10^6	1.9×10^4	1.8×10^5	1.7×10^5
6	1.5×10^6	1.1×10^6	8.2×10^5	2.0×10^4	4.0×10^5	4.3×10^5
7	1.9×10^6	1.4×10^6	8.3×10^5	2.1×10^4	6.8×10^5	5.9×10^5
8	2.6×10^6	1.6×10^6	1.3×10^6	2.1×10^4	1.8×10^6	1.7×10^6
9	1.5×10^6	9.2×10^5	6.0×10^5	1.1×10^4	1.1×10^6	1.2×10^6
10	1.6×10^6	1.0×10^6	9.8×10^5	1.4×10^4	8.7×10^5	7.9×10^5

Note: The sample material consisted of clay loam soil (characterized as being 22% sand, 51% silt, and 27% clay) that contained sodium fluoride at the noted concentration. The positive control contained the growth medium solution, the algal cell inoculum, and zinc chloride (128 µg/l). The negative control was prepared by adding the algal cells to an undiluted aliquot of growth medium. At the end of a 96-hour test, the negative control sample should range from 1.6×10^6 to 2.6×10^6 cells/ml.

APPENDIX TABLE XI. ALGAL CELL CONCENTRATION DETERMINED AT 96-HOUR INTERVAL IN RESPONSE TO ELUATE FROM 48.0 mg/g SODIUM FLUORIDE SOIL SAMPLES (All Values, Including 96-Hour Response for Negative and Positive Controls, Given as Cell Number/ml)

Assay Number	Growth Media	Eluate Dilution 1%	Eluate Dilution 10%	Eluate Dilution 80%	Positive Control #1	Positive Control #2
1	2.5×10^6	1.8×10^6	6.5×10^5	1.5×10^4	9.7×10^5	8.9×10^5
2	2.7×10^6	1.8×10^6	6.1×10^5	1.5×10^4	7.7×10^5	8.6×10^5
3	2.9×10^6	2.4×10^6	1.1×10^6	1.9×10^4	1.6×10^6	1.6×10^6
4	2.8×10^6	2.5×10^6	9.3×10^5	1.7×10^4	1.3×10^6	1.2×10^6
5	3.2×10^6	2.6×10^6	9.0×10^5	2.1×10^4	1.5×10^6	1.5×10^6
6	3.0×10^6	2.6×10^6	6.0×10^5	1.5×10^4	1.5×10^6	1.6×10^6
7	3.0×10^6	2.3×10^6	4.4×10^5	1.5×10^4	1.4×10^6	1.4×10^6
8	2.9×10^6	2.9×10^6	1.1×10^5	1.7×10^4	1.6×10^6	1.5×10^6
9	2.2×10^6	2.3×10^6	1.0×10^6	2.2×10^4	1.5×10^6	1.5×10^6
10	3.2×10^6	2.8×10^6	1.1×10^6	1.8×10^4	1.5×10^6	1.6×10^6

Note: The sample material consisted of clay loam soil (characterized as being 22% sand, 51% silt, and 27% clay) that contained sodium fluoride at the noted concentration. The positive control contained the growth medium solution, the algal cell inoculum, and zinc chloride (128 µg/l). The negative control was prepared by adding the algal cells to an undiluted aliquot of growth medium. At the end of a 96-hour test, the negative control sample should range from 1.6×10^6 to 2.6×10^6 cells/ml.

APPENDIX TABLE XII. ALGAL CELL CONCENTRATION DETERMINED AT 96-HOUR INTERVAL IN RESPONSE TO ELUATE FROM 60.0 mg/g SODIUM FLUORIDE SOIL SAMPLES (All Values, Including 96-Hour Response for Negative and Positive Controls, Given as Cell Number/ml)

Assay Number	Growth Media	Eluate Dilution 1%	Eluate Dilution 10%	Eluate Dilution 80%	Positive Control #1	Positive Control #2
1	1.2×10^6	6.7×10^5	2.1×10^5	9.4×10^3	4.0×10^5	4.3×10^5
2	1.9×10^6	8.9×10^5	4.7×10^5	1.3×10^4	6.4×10^5	7.6×10^5
3	1.9×10^6	7.9×10^5	4.7×10^5	1.2×10^4	6.4×10^5	7.6×10^5
4	3.1×10^6	2.8×10^6	8.0×10^5	1.8×10^4	3.3×10^5	4.3×10^5
5	2.0×10^6	1.4×10^6	5.1×10^5	1.3×10^4	1.3×10^6	1.6×10^6
6	2.8×10^6	2.8×10^6	8.1×10^5	1.1×10^4	1.1×10^6	1.5×10^6
7	1.8×10^6	1.5×10^6	4.7×10^5	2.0×10^4	1.2×10^6	1.2×10^6
8	1.7×10^6	5.6×10^6	4.0×10^5	2.9×10^4	5.7×10^4	4.7×10^4
9	2.4×10^6	2.2×10^6	6.8×10^5	1.6×10^4	5.1×10^4	4.8×10^4
10	1.8×10^6	2.0×10^5	6.1×10^5	2.0×10^4	7.3×10^5	6.9×10^5

Note: The sample material consisted of clay loam soil (characterized as being 22% sand, 51% silt, and 27% clay) that contained sodium fluoride at the noted concentration. The positive control contained the growth medium solution, the algal cell inoculum, and zinc chloride (128 µg/l). The negative control was prepared by adding the algal cells to an undiluted aliquot of growth medium. At the end of a 96-hour test, the negative control sample should range from 1.6×10^6 to 2.6×10^6 cells/ml.

APPENDIX B

METHOD PROTOCOL

(CHLOROPHYTA ASSAY USING SOIL AS SAMPLE MATERIAL)

I. INTRODUCTION

This procedure is used to assess the potential toxicity of soil samples such as might be collected in the vicinity of hazardous waste sites. Deionized water is added to individual soil samples and the resulting eluate is tested for toxicity using freshwater algae (Selenastrum capricornutum).

The algal assay component of this test has been derived from a well established freshwater assay used to determine algal growth inhibition and stimulation. Several previous efforts have been directed toward the standardization of this original algal method (International Organization for Standardization, 1987; Standard Methods, 1985; ASTM, 1985; Federal Register, Vol. 50, #188, 1985). A variation of this algal procedure has recently been used (EPA-Corvallis) as a screening technique for soil eluates and was found to be of considerable value when assessing potential ecological hazards at various waste site locations.

This protocol provides instructions for conducting the Chlorophyta assay when soil is used as the initial sample material and when the procedure is used primarily as a screening technique. Because an aqueous eluate is initially acquired from the original soil sample and because this eluate is filtered and diluted prior to toxicity testing, the procedure does not address non-water soluble chemicals nor does it test for the presence of volatile compounds. In addition, procedures associated with the collection of soil samples (e.g., representative sampling of a waste site area) are not addressed. Based on the ecological importance of unicellular algae, however, this relatively simple technique can provide an environmental hazard assessment for those chemicals that are, in fact, the compounds most likely to be transported to surface and ground waters.

II. OVERVIEW OF TEST PROCEDURE

A soil sample is collected and taken to the laboratory where water is added to the soil and a leachate is eventually collected. The leachate (soil eluate) is then filtered and pH adjusted prior to testing. The algal culture (Selenastrum capricornutum) is maintained routinely by the testing laboratory and algal growth media are also routinely prepared.

The soil eluate is diluted to a series of three separate concentrations using the growth media preparation. Algal cells are transferred from the stock culture to each dilution flask. A negative control also is prepared using the growth media preparation and the algal cell addition. Zinc chloride is added in addition to the growth media and algal cells when preparing the positive control. Ninety-six hours after the flasks are inoculated, the cell concentration in each flask is determined using a coulter counter.

Duplicate flasks are routinely prepared for the negative control, positive control, 80% soil eluate, 10% soil eluate, and 1% soil eluate -- i.e., a total of 10 separate flasks. An average cell count is taken at 96 hours for the negative control, 80% soil eluate, 10% soil eluate, and 1% soil eluate. In the case of the positive control, separate response values are tabulated.

The test result requires a two stage calculation. The first step is to determine the eluate effect for each of the three dilutions and the second step is to extrapolate an EC_{50} value that serves as the final test result. The eluate effect is tabulated using the initial inoculum cell count and the 96-hour cell count determined for each eluate dilution and for the negative control. The proportion of growth media used in a given dilution flask also is included in the tabulation. A positive control response also is routinely calculated for each separate assay.

III. DEFINITIONS

A. Sample Material

The sample consists of 125 grams of air dried soil. However, at least 500 grams of soil should probably be taken at time of sample collection.

B. Incubator

An incubator consists of a growth chamber containing certain controls for temperature, relative humidity, and lighting. The only real requirement is that the incubator unit provide the necessary controlled environment.

C. Culture Flasks

Culture flasks are 125-ml Erlenmeyer flasks containing growth media that are stoppered with foam plugs. The flasks and plugs are sterilized using an autoclave prior to use.

D. Inoculum

The inoculum refers to the algal cells that will be added to an assay flask at the start of a test. The addition of cells should contain a sufficient number of cells so that the resulting cell concentration (in the 50 ml of soil eluate plus growth medium) will be $1 \times 10^4 \pm 1 \times 10^3$ cells/ml.

The algal cells will have been transferred to the inoculum flask from a 7-day-old parent stock culture maintained in a culture flask with 50 ml of growth media. The inoculum flask should contain 50 ml of previously sterilized deionized water when the algal cells are transferred.

Cell concentrations in the stock culture flask should be approximately 4.2×10^6 cells/ml with a total cell number of approximately 2.1×10^8. The actual cell concentration present in the stock culture is determined and a portion of these cells are transferred to the inoculum flask as tabulated below.

$$\text{culture cells transferred to inoculum flask (volume of culture transferred)} = \frac{\text{volume of inoculum flask contents (ml)} \times \text{cell concentration needed for inoculum flask (cells/ml)}}{\text{concentration of cells in stock culture (cells/ml)}}$$

example:

volume of inoculum = 50 ml sterilized deionized water

cells needed for inoculum flask = assume 1.0×10^6 cells/ml

concentration of cells in stock culture = assume 4.2×10^6 cells/ml

therefore

$$\frac{50 \text{ ml} \times 1 \times 10^6 \text{ cells/ml}}{4.2 \times 10^6 \text{ cells/ml}} = \frac{5.0 \times 10^7}{4.2 \times 10^6}$$

= 11.9 ml of culture cells to be transferred to the inoculum flask.

IV. EQUIPMENT AND SUPPLIES

A. Equipment

1. Incubator - growth chamber

   The incubator should provide for continuous illumination using cool-white fluorescent lighting at 4300 ± 430 lumens and should be capable of maintaining a 24 ± 2°C temperature (Unitherm Corporation or equivalent).

2. Fumehood

3. Balance

4. pH meter

   One or more pH meters may be required to determine the pH of sample eluate dilutions and assay controls

5. Shaker - agitator

   A shaker, or shaker-incubator unit, is used to ensure aeration of algal cultures. A variable stroke shaker should probably be used that is capable of at least 120 excersions/minute with a suitable platform for the 125-ml flasks (American Scientific Products or equivalent).

6. Particle counter

   A variety of electronic particle counters are available to determine cell concentrations (cells/ml). The counter should be capable of counting particles up to 40 μm, should have an adjustable threshold scaling circuit, and should have a 100 μm aperture (Coulter Electronics Inc. or equivalent).

7. Dilution vials

   Twenty-ml, capped, plastic vials (10 vials per assay) are needed for use with the particle counter (Fisher Scientific or equivalent).

8. Media dispensers

   Media dispensers are suggested for a quantitative transfer of dilutents and calibration solutions into dilution vials when calibrating the particle counter.

9. Refrigerated centrifuge

A refrigerated centrifuge must be available that is capable of maintaining a 4°C temperature at 10,000 RPM for 10 minutes. The centrifuge head should allow for at least eight 50-ml tubes, i.e., enough for one complete assay (International Equipment Co. or equivalent).

10. Magnetic stirrers and stirring bars

11. Refrigerator

12. Water purity system

The water purity unit selected should provide either (a) double deionized, (b) reverse osmosis deionized, or (c) distilled-deionized water.

13. Ultrafiltration system

The ultrafiltration sequence must achieve a 0.45 μm filtrate from the original soil eluate. Several nonsterile prefilter steps may be necessary for some of the samples. Typically, a 0.7 μm and a 0.45 μm filtering step will be sufficient. However, some of the soil eluates may contain a precipitate, and prefilter steps of 2.7 μm, 1.6 μm, and/or 1.0 μm will be necessary in addition to the 0.7 μm and 0.45 μm filters.

The reverse osmosis-deionized water can be sterilized adequately either by autoclaving or by using a 0.45 μm filter.

14. Thermograph

Either a 7-day continuous recording thermograph or an electronic thermograph should be available (Weathertronics Corporation or equivalent).

15. Autoclave

A steam autoclave capable of wet sterilization and dry vacuum sterilization should be available to obtain sterile glassware and sterile water. Some procedure, such as sterility indicator tape, should be used to verify that sterilization has been achieved.

16. Sterile chamber (ultraviolet)

Two UV sterile chambers are used. Typically, one chamber is used when preparing the dilutions and will contain a magnetic mixer and a burner (touch-o-matic) for sterilization. The second chamber is used for inoculating the test flasks. These chambers can be constructed from 2 in. by 2 in. lumber that is covered with aluminum foil on the interior and with approximately four Mil plastic sheeting on the outer surface. Two UV lights (GE/G15T8 germicidal 15 watt) are suspended 24 in. from the surface to provide continuous lighting. (Note: Mil is a thickness designation for plastic film.)

17. Vacuum pump

Vacuum pumps are required to vacuum filter the soil eluate. There should be an in-line vacuum flask to capture any accidental vacuuming of the eluate and a pump gauge should be present and routinely checked.

18. Blender

A Waring blender with a stainless steel container is used for processing the soil to an essentially uniform particle size prior to drying (Fisher Scientific or equivalent).

B. Supplies

1. Algal growth media (Table I)

The algal growth media consists of solutions of inorganic chemicals in purified and sterilized water (Certified A.C.S. grade or equivalent).

2. Centrifuge tubes

Eight 50-ml centrifuge tubes are used to provide 400 ml of soil eluate per assay.

3. Filter paper

As was mentioned previously (i.e., ultrafiltration system), several filtrations of decreasing pore size may be required depending on the characteristics of the centrifuged soil eluate (Whatman glass microfiber 2.7 μm [GF/D]; 1.6 μm [GF/A]; and/or 1.0 μm [GF/B]). Typically, only two filtering steps are needed. Initially, a nonsterile 0.7 μm glass fiber filter (Whatman glass microfiber [GF/F]) is used followed by a 0.45 μm nylon filter (MSI Micronsep Magna). A millipore filter holder can be used with the 0.45 μm sterile filter.

4. Autoclave tape

In some instances, sterilization monitor strips or indicator tape should be used to verify that sterilization conditions have been achieved. Sterile wraps are used subsequently to seal the flasks.

5. Glassware (sufficient for conducting multiple assay)

4	-	2000 ml Erlenmeyer flasks
10	-	1000 ml Erlenmeyer flasks
2	-	500 ml Erlenmeyer flasks
200	-	125 ml Erlenmeyer flasks
5	-	1000 ml graduated cylinders
4	-	500 ml graduated cylinders
4	-	100 ml graduated cylinders
6	-	50 ml graduated cylinders
6	-	500 ml beakers
12	-	1000 ml volumetric flasks with stoppers
6	-	1000 ml side arm vacuum flasks
4	-	500 ml side arm vacuum flasks
1	-	5000 ml Oxford pipette
12	-	micropipettes (1 μl to 1,000 μl)

6. Thermometer

Mercury thermometers are used that will indicate temperatures from -10°C to 60°C in 1° increments. Maximum-minimum thermometers that register from -40°C to 50°C are also recommended.

7. Buffer solutions

Three pH buffer solutions (pH 4, pH 7, and pH 10) are required.

8. Isotonic solution

An isotonic, azide free, solution is suggested for use with the particle counter. However, a sterilized (0.45 μm filter), one percent sodium chloride solution can be prepared in the laboratory and used instead of the commercial preparation (Azide Free Isotone II; American Scientific Products or equivalent).

9. Ceramic pan

Ceramic or pyrex glass pans of approximately 37 cm x 26 cm with a 6 cm rim are recommended for use in drying the soil samples.

10. Teflon mixer (spoon/spatula)

11. Teflon containers

Twenty five (25) 1-liter, wide mouth, capped, teflon containers are suggested.

12. Sodium hydroxide (analytical grade)

13. Hydrochloric acid (analytical grade)

14. Zinc chloride (analytical grade)

15. Acetone (reagent grade)

16. Detergent (nonphosphate)

V. PREPARATION OF SUPPLIES AND EQUIPMENT

A. Glassware

1. Washing and rinsing

Glassware should be thoroughly cleaned and be free of contamination prior to the assay.

a. rinse with tap water immediately after use

b. soak and clean in nonphosphate detergent

c. rinse with tap water a minimum of three times

d. rinse with acetone

e. rinse with 10 percent hydrogen chloride

f. rinse three times with tap water

g. rinse three times with reverse osmosis-deionized water

2. Sterilizing

Glassware used during the assay, i.e., stock solution glassware, assay flasks, foam plugs used with flasks, etc. must be sterilized (autoclave) after the washing sequence.

B. Ultrafiltration

To provide sterile filtration for the algal growth media and the various assay solutions, it is suggested that the laboratory use an autoclavable porcelain Buchner funnel attached to a one liter side arm flask that is, in turn, connected to a vacuum pump. A 0.45-μm sterile glass fiber filter is used with this unit.

C. Water

The reverse osmosis-deionized water, used for the media preparation, is sterilized either by autoclaving or by ultrafiltration with a 0.45 μm or 0.22 μm filter. The pH of the sterile water should be checked periodically and must be maintained between pH 6 and pH 8.

VI. ASSAY PROCEDURE

A. Preparation of Sample Material

Approximately 500 grams of soil are collected and sent to the laboratory for testing. The soil is transferred immediately to a ceramic or pyrex pan (approximate dimensions: 37 cm x 26 cm with a 6 cm rim) and placed in a fume hood and allowed to air dry for 24 hours. A blender initially is used to homogenize the soil and care should be taken to stir and smooth the soil (even layer) in each ceramic pan. Any rocks or gravel present in the sample are manually removed prior to drying.

After 24 hours, the soil is removed from the fume hood and a 125-gram portion is transferred to a 1-liter teflon container. Five hundred ml of deionized water are added to the sample. The teflon container is then capped and mechanically shaken in the dark for 48 hours (approximately 120 EPM) at room temperature. Exactly 125 grams of soil and 500 ml of deionized water should be added because relatively modest variations in this water addition can affect the final test result.

At the end of 48 hours, the shaker is stopped and the container is allowed to sit for approximately 10 minutes. The liquid is decanted from the container and transferred to 50-ml centrifuge tubes. The eluate is centrifuged (10,000 rpm for 10 minutes) at 4°C. The total amount of soil eluate recovered from the teflon container will vary somewhat between samples but at least 400 ml should be available.

As mentioned previously, several preliminary filtering steps (i.e., 2.7 μm, 1.6 μm, 1.0 μm, etc.) may be required depending on the characteristics of any precipitate noted in the resulting soil eluate.

After centrifugation, however, the eluate typically is transferred to a Buchner funnel that contains a 0.7-μm nonsterile glass fiber filter and filtered under vacuum. The eluate subsequently is filtered through a 0.45-μm nylon filter to obtain a sterile eluate.

The eluate dilution steps are conducted using the 100% solution of growth media. Three concentrations are prepared that consist of (a) 1 ml of eluate and 99 ml of growth media, (b) 10 ml of eluate and 90 ml of growth media, and (c) 80 ml of eluate and 20 ml of growth media. The resulting dilutions are designated as 1% soil eluate, 10% soil eluate, and 80% soil eluate, respectively. Care should be taken to ensure thorough mixing of soil eluate and the added growth media. In addition, a consistent dilution series (1%, 10%, and 80%) must be used from one assay to another. After the eluate is diluted with the solution of growth media, the algal cells are then added for testing. (Note: During an actual assay duplicate flasks are routinely prepared and tested for the negative control, positive control, 80% soil eluate, 10% soil eluate, and 1% soil eluate -- i.e., a total of 10 flasks. An average cell count ultimately is taken for the negative control, 80% soil eluate, 10% soil eluate, and 1% soil eluate, respectively. In the case of the positive control, separate response values are tabulated.)

B. pH Adjustments

Although it has previously been established that the growth response of Selenastrum capricornutum is not affected over a wide range of pH values, the potential array of chemicals and soil types encountered when using this procedure occasionally will require pH alternations for the leachate. Consequently, several pH determinations must be routinely conducted as part of the assay procedure and, when required, pH adjustments are made using 1N sodium hydroxide and 1N hydrochloric acid.

When the soil eluate is first collected, the pH should be adjusted to between 6.5 and 8.5. Immediately prior to the sterile filtration step, the eluate pH is checked again and should again be between 6.5 and 8.5. When the assay is concluded at the 96-hour time interval, the contents of each dilution flask and of each control flask must be between pH 6.0 and 9.0. For a given elute dilution to be used in the final EC_{50} tabulation, the pH value for both replicates (at a given concentration) must be within this pH range. In addition, the pH value for the control samples also must be within this range in order for the final result to be considered as a valid test response.

Although pH adjustments are not typically required for the deionized water and for the growth media preparation, these materials should be maintained between pH 6.0 and 8.0 for the water and between pH 7.0 and 8.0 for the growth media. If the deionized water and the growth media

are kept within these pH ranges, only slight adjustments, if any, should be required for the subsequently prepared assay flask material.

C. Preparation of Growth Media

The growth medium is prepared by adding 1.0 ml of each of the five nutrient solutions (macronutrient and micronutrient solutions shown in Table I) to 900 ml of sterilized deionized water (either filter sterilized or autoclaved). After each addition, the composite solution should be mixed thoroughly . The final composite volume is brought to 1 liter with sterilized deionized water. The growth medium should be prepared as needed; however, the composite preparation can be stored in the dark at 4°C for several weeks. As mentioned previously, the pH of the growth medium should be between 6 and 8.

D. Maintenance of Algal Culture

A starter culture of Selenastrum capricornutum can be obtained from the American Type Culture Collection (ATCC#22662). The frozen starter culture is warmed in a water bath at 35°C for 2 minutes and then aseptically transferred to a 125-ml culture flask that contains 5 ml of growth medium. After 48 hours, an additional 5 ml of growth medium are added.

Seventy-two hours after the initial transfer, 35 ml of growth medium are again added and the culture is grown for an additional 2 days. The culture then is transferred and incubated for at least another 5 days or until the culture reaches 2.5×10^6 cells/ml.

This initial algal culture can be used to inoculate several flasks of growth medium. Aseptic conditions must routinely be followed to ensure the maintenance of a monoculture. The stock culture is maintained at 24 ± 2°C under continuous lighting. The lighting is kept at approximately 4300 lumens and the flasks are agitated at 100 EPM. The foam plugs used with each flask must not become wet as a result of the constant agitation.

The stock culture should be routinely transferred on a 7-day basis in order to achieve an essentially logarithmic phase of growth at time of cell transfer and to provide adequate cells for the various assays. The cell count of a stock culture at 7 days must reach at least 3.8×10^6 cells/ml. Aseptic transfer of 1 ml from a 7-day-old culture to 50 ml of growth medium usually is sufficient. The algal culture can be maintained for longer periods of time using standard streaking transfer techniques. The cells are transferred with a wire loop onto a sterile 1% agar slant (prepared using the algal growth medium). The agar slants then are placed with the stock cultures until algal colonies appear. Subsequent stock cultures can be started using a sterile loop to scrape cells from the agar which are then transferred

to 50 ml of liquid growth media. New stock cultures should be started every 30 days by removing a health colony from the agar plate.

E. Preparation of Sample Dilutions

The soil eluate is monitored immediately after its collection from the soil sample and is checked again immediately prior to the sterile filtration step. After the sterile filtration step, the eluate is diluted with the growth media. The dilution sequence consists of 3 flasks containing 1 ml of eluate and 99 ml of growth medium, 10 ml of eluate and 90 ml of growth medium, and 80 ml of eluate and 20 ml of growth medium, respectively. Care must be taken to ensure that a consistent dilution series of 1%, 10%, and 80% is used from one assay to another.

The flasks are inoculated with algal cells to achieve a cellular concentration of $1 \times 10^4 \pm 1 \times 10^3$ cells/ml (i.e., agitate the cell suspension, count an aliquot of cells using the cell counter, make a calculation to determine the required aliquot volume, and transfer cells to various flasks using a pipette). The flasks then are incubated for 96 hours at 24°C ± 2°C with continuous lighting (4300 ± 430) and continuous agitation (100 EPM). The pH must be determined for each flask after the 96-hour interval. If the concluding pH is not between 6.0 and 9.0, the assay response for that particular flask is not used when tabulating the final test result. As stated previously, two flasks are prepared at each dilution and the average cell count is taken for purposes of response tabulation at the end of 96 hours.

At the end of the test, the flasks are agitated and an aliquot is taken and diluted for purposes of cell counting. A 0.5-ml aliquot is taken from each flask and placed in a vial (disposable blood dilution vial). A 19.5-ml addition of an electrolyte medium is added to obtain a 40-fold dilution (either Isoton II or another medium recommended for use with a Coulter counter). The electronic particle counter, equipped with the recommended 100 μm aperture for counting algal cells, will analyze a 0.5-ml sample with a 13-second count. After counting the sample three times and subtracting the background counts, an average sample count is tabulated and the value is multiplied by 40 (to correct for the dilution factor of 1:40). This subsequent value is then doubled to achieve the number of cells per ml (i.e., the Coulter Counter provides a reading based on a 0.5 ml sample).

TABLE I. COMPOSITION OF ALGAL GROWTH MEDIA

MACRONUTRIENTS			
Stock Solution*		Nutrient Composition Prepared Medium	
Compound	Concentration (g/l)	Element	Concentration (mg/l)
STOCK SOLUTION I			
$NaNO_3$	25.500	N	4.200
$NaHCO_3$	15.000	Na**	11.001
		C	2.143
STOCK SOLUTION II			
K_2HPO_4	1.044	K	0.469
		P	0.186
STOCK SOLUTION III			
$MgSO_4$ $7H_2O$	14.700	S	1.911
STOCK SOLUTION IV			
$MgCL_2$ $6H_2O$	12.164	Mg***	2.904
$CaCl_2$ $2H_2O$	4.410	Ca	1.202

MICRONUTRIENTS			
Stock Solution		Nutrient Composition Prepared Medium	
Compound	Concentration (mg/l)	Element	Concentration (μg/l)
STOCK SOLUTION V			
H_3BO_3	185.520	B	32.460
$MnCl_2$ $4H_2O$	415.610	Mn	115.374
$ZnCl_2$****	3.271	Zn	1.570
$CoCl_2$ $6H_2O$****	1.428	Co	0.354
$CuCl_2$ $2H_2O$****	0.012	Cu	0.004
Na_2MoO_4 $2H_2O$****	7.260	Mo	2.878
$FeCl_3$ $6H_2O$	160.000	Fe	33.051
Na_2EDTA $2H_2O$	300.000	--	--

*Other forms of the salt may be used if the resulting element concentrations are the same as those listed.

**Includes Na from $NaNO_3$.

***Includes Mg from $MgSO_4$ $7H_2O$.

****Prepare at 100 times concentration and add in designated amount to stock solution V.

F. Preparation of Control Samples

1. Negative control

The negative control is prepared by adding the algal cells to an undiluted aliquot of growth media. A 50-ml aliquot of growth medium is transferred aseptically to each of two sterile, 125-ml Erlenmeyer flasks. The algal cells are then added to achieve a final dilution of $1 \times 10^4 \pm 1 \times 10^3$ cells/ml. At the end of a 96-hour test, the negative control sample should range from 1.6×10^5 to 2.6×10^5 cells/ml. The cell concentration must be at least 1.0×10^6 at the 96-hour interval in order to obtain a valid test result for the overall assay.

2. Positive control

The purpose of the positive control is to provide information on whether the algal assay is responding to a known toxic material and, after several assays have been conducted, the various positive control responses can provide data that are used to assess the variability occurring between assays. The positive control response is not used when tabulating the EC_{50} value for the final test result; but, the positive control values obviously are used to help determine whether a test response is accepted as a valid test result.

The positive control contains the growth media solution, the algal cell inoculum, and zinc chloride. Two positive control flasks are prepared for each assay. The initial algal cell concentration should be $1 \times 10^4 \pm 1 \times 10^3$. The average response of the two positive controls must show at least a 15% inhibition as compared to the negative control. In addition, the cell counts from the two positive control flasks must be within 50% of each other in order to achieve a valid test result. If these conditions do not exist for the positive control result, the assay results will not constitute a valid test response. The suggested zinc chloride concentration is 128 μg/l but the suitability of this concentration should be verified by the testing laboratory prior to beginning an actual series of assays (Appendix Tables I, II, and III).

VII. TABULATION OF TEST RESULT

The number of algal cells/ml in a given flask may have increased, decreased, or remained constant compared to the negative control. As mentioned previously, several conditions must exist in order for a test result to be considered as valid. The negative control flask must achieve a cell count of at least 1.0×10^6 cells/ml by the 96-hour time interval. An average of the two positive control flasks must also show at least a 15% cell growth inhibition

(compared to the negative control) and the two positive control results must be within 80% of each other. For a given eluate dilution to be used in the final EC_{50} tabulation, the pH value for at least one of the replicates (at a given concentration) must be between pH 6.0 and 9.0.

A final test result requires a two-stage calculation. The first step is to determine the eluate effect for each of the three dilutions and the second step is to extrapolate an EC_{50} value which serves as the final test result.

The eluate effect is tabulated from the initial inoculum cell count and from the final 96-hour cell count for each separate dilution and for the negative control, respectively. The positive control result, not directly used in the tabulation, is based also on an initial inoculum cell count and on a subsequent 96-hour cell count. The effect of the soil eluate on algal growth is tabulated by using the following information. The negative control (NC), positive control (PC), 80% soil eluate (E_{80}), 10% soil eluate (E_{10}), and 1% soil eluate (E_1) values each represent an average (cells/ml) for each of two flasks. The inoculum (IN) represents the concentration of cells in a given flask at the start of an assay and the proportion of growth media (G) used in a given flask will depend on the amount of soil eluate being tested.

NC = cells/ml for negative control; must be at least 1.0×10^6 cells/ml (96 hours)

PC = cells/ml for positive control (96 hours)

E_{80} = cells/ml for 80% eluate and 20% growth media (96 hours)

E_{10} = cells/ml for 10% eluate and 90% growth media (96 hours)

E_1 = cells/ml for 1% eluate and 99% growth media (96 hours)

IN = cells/ml in flask immediately after addition of algal cells; value should be $1 \times 10^4 \pm 1 \times 10^3$ (start of assay)

G = proportion of growth media used; the value will be either 0.20 for the 80% soil eluate, 0.90 for the 10% soil eluate, or 0.99 for the 1% soil eluate. (start of assay)

$$\text{Effect of Eluate } (E_{80} \text{ for example}) = \frac{(E_{80} - IN) - G(NC-IN)}{G(NC-IN)}$$

The effect can result in either a negative or a positive value. A negative value indicates a decrease in cell number i.e., an inhibiting effect compared to the negative control. A positive value indicates an increase in cell number compared to the negative control. For purposes of the above calculation, any positive value represents a stimulatory effect. If the positive value is 10 or

less, the value is reduced to zero and zero is used for the respective dilution when plotting the concentration/response curve for the EC_{50} determination. Retention of values that are ten or less represents a statistical correction to include random variation around the zero response. If the positive value is greater than ten, the value is not used when calculating the EC_{50}.

To calculate the positive control effect, a similar tabulation is used. It is assumed that G (proportion of growth media) will be a value of 1.0 since no actual eluate is used and only a small volume (μl) of the zinc chloride solution is added.

$$\text{Effect of Positive Control} = \frac{(PC\text{-}IN) - G(NC\text{-}IN)}{G(NC - IN)}$$

The EC_{50} value for a given soil sample is determined by plotting the calculated effect (%) versus the various tested eluate dilutions (concentrations). The plotted line can simply be drawn to an apparent best fit or, preferably, a regression calculation can be used. If a regression calculation is used, the data should still be plotted on a graph for visual verification. The final test result should always include a brief statement that identifies the original soil collection. Assume that, for a given soil sample, the EC_{50} concentration is calculated to be a 75% eluate dilution. The brief accompanying statement should (1) identify where the soil was collected, (2) mention that the 100% eluate was acquired from a one part air dried soil with a four part water addition, and (3) state that a 75% dilution of this eluate resulted in a 50% inhibition in the 96-hour growth of Selenastrum capricornutum.

APPENDIX: POSITIVE CONTROL EVALUATION AND ALTERNATE METHODS OF DATA TABULATION

Positive Control

Several concentrations of the positive control compound should be tested prior to beginning a series of assays. This is suggested in order to verify the dose/response curve for zinc chloride (or any other compound selected for use as a positive control). Appendix Table I shows a series of zinc chloride concentrations and the resulting 96-hour algal response. Based on this series of concentrations, the EC_{50} for sodium chloride was determined to be 128.3 μg/l. The positive control flasks, therefore, were prepared at a 128 μg/l zinc chloride concentration.

A series of positive control assays were also conducted to verify the expected variability between different assays as well as the variability between control flasks during a single assay. The results are shown in Appendix Table II and III for 128 μg/l and for 74 μg/l of zinc chloride, respectively. As mentioned above, the suggested positive control concentration is 128 μg/l.

Based on the Table II assays, one could reasonably expect the 128 μg/l zinc chloride to produce a growth inhibiting effect of between -90.6 and -99.8 when

tabulated using the given formula. In addition, the variation between the two positive control assays (%) should not be greater than 4.6. The EC_{50} determinations are not required on a routine basis (for each assay) since a series of different concentrations would be required.

Alternate Methods of Data Tabulation

Algal Response to Eluate

The following equation has already been given in the protocol.

$$E_{80} = \frac{(E_{80} - IN) - G(NC-IN)}{G(NC-IN)}$$

If there is a decrease in algal growth, the above tabulation will result in a negative value. If a logarithmic transformation is planned during data analysis, a positive value can be achieved using the following equation.

$$E_{80} = \frac{G(NC - IN) - (E_{80} - IN)}{G(NC-IN)}$$

Variation Between Positive Controls

To approximate the difference (%) between two positive control values at the end of 96 hours, one approach would be to determine the cell count for both flasks and then to divide the greater count into the lesser count as shown below.

$$\text{difference (\%) between the two positive controls} = \left(1 - \frac{\text{lesser cell count}}{\text{greater cell count}}\right) \times 100$$

However, this approach is not really applicable to the other requirement for a positive control value i.e., to allow for a comparison over weeks or months in the assay's responsiveness to a given toxic material. The negative control and the initial cell concentration are obviously required for a more complete determination.

TABLE II. CHLOROPHYTA RESPONSE TO VARIOUS CONCENTRATIONS OF ZINC CHLORIDE (Positive Control Compound Added Directly to Assay Flask)

Zinc Chloride Concentration (µg/l)	Algal Cells/ml After 96 Hours	Calculated Effect Value
negative control	3.1×10^6	---
9.7	3.1×10^6	0.3
18.5	3.1×10^6	- 0.1
37.0	3.0×10^6	- 5.0
74.0	2.4×10^6	- 24.4
148.0	1.2×10^5	- 96.5
296.0	1.3×10^4	- 99.9

* $$\text{Response to Positive Control} = \frac{(PC - IN) - G(NC - IN)}{G(NC - IN)}$$

NC = cells/ml for negative control
PC = cells/ml for positive control
G = proportion of growth media used; assumed to be 1.0 since no actual eluate is used and only a small volume (µl) of zinc chloride solution is added
IN = cells/ml in flask immediately after addition of algal cells

** Based on this series of concentrations, the calculated EC_{50} for sodium chloride is 128.3 µg/l. As stated previously, the positive control compound is added directly to an assay flask and not to a soil sample.

TABLE III. SERIES OF ALGAL ASSAYS USING 128 µg/l OF ZINC CHLORIDE (Two Separate Assays are Conducted with Positive Control Compound Added Directly to Assay Flask)

Positive Control Response

Assay Number	Calculated Effect I	Calculated Effect II	Variation Between Assays (%)
1	- 92.2	- 89.9	2.5
2	- 94.6	- 96.9	2.4
3	- 95.6	- 97.1	1.6
4	- 96.3	- 94.9	1.5
5	- 85.7	- 83.4	2.7
6	- 96.3	- 97.7	1.4
7	- 97.9	- 94.6	3.3
8	- 89.9	- 98.9	9.2
9	- 96.9	- 96.7	0.1
10	- 98.0	- 98.4	0.4
11	- 96.9	- 96.1	0.8
12	- 98.0	- 97.4	0.6
X	- 94.8	- 95.2	2.2
±SD	3.8	4.4	2.4
CV (%)	4.0	4.6	---

TABLE IV. SERIES OF ALGAL ASSAYS USING 74 μg/l OF ZINC CHLORIDE
(Two Separate Assays are Conducted with Positive Control Compound Added Directly to Assay Flask)

Assay Number	Positive Control Response: Calculated Effect I	Positive Control Response: Calculated Effect II	Variation Between Assays (%)
1	- 45.0	- 48.3	6.9
2	- 52.0	- 50.6	1.9
3	- 53.0	- 59.3	10.7
4	- 51.0	- 69.0	26.1
5	- 54.2	- 62.2	12.9
6	- 58.8	- 50.2	14.7
$\bar{X}$ =	- 52.3	- 56.6	
± SD =	4.5	8.2	
CV (%) =	8.6	14.5	

APPENDIX C

CRITICISM OF WRITTEN METHOD PROTOCOL

MADE DURING INITIAL CONSENSUS

REVIEW AND/OR DURING FINAL

EVALUATION REVIEW

Criticism: Too much latitude is allowed in the pH range required for the eluate dilution flasks.

A more limited pH range for the eluate dilution flasks is probably a valid criticism; however, there are several reasons why this was not a requirement as it has been in several other versions of this basic algal assay. The filtered soil eluate will contain some compounds and elements in addition to any pollutants present and, perhaps more significantly, these leached soil chemicals will vary depending on the type of soil sampled, the amount of humic material present, the existing soil pH, etc. During the current study, a more closely controlled pH value seemed to result in a more frequent formation of eluate precipitates (different types of soils were used during initial phases of evaluation).

As expected, the pH had a definite effect on the final assay result during the ruggedness evaluation and whatever the final decision concerning pH limits, the written protocol instructions will need to be strictly followed. However, as was discussed by the reviewers, the latitude in pH probably contributes to the overall poor method sensitivity noted during the current evaluation and to variations in the precision determination when using the organic compound 2,4-dichlorophenol.

Perhaps additional method testing should be done specifically to select the most suitable pH range (i.e., a type of method optimization testing) using various soil types that contain different amounts of organic matter and different concentrations of pollutant compounds.

Criticism: The source of algal cells should be emphasized and some discussion should be presented concerning recent taxonomic revisions for this particular species.

Initial algal cultures are obtained from the American Type Culture Collection (ATCC #22662) and this source is a suggested supplier of starter cells when using the assay. The cells are listed as Selenastrum capricornutum and, while of no real significance to the current evaluation, the taxonomic scheme apparently continues to be as follows: Phylum: Chlorophyta; Class: Chlorophyceae; Order: Chlorococcades; Family: Oocystaceae; Genus: Selenastrum; Species: Selenastrum capricornutum.

Criticism: The cell concentration required for the negative control flask is not sufficient and additional cell count determinations should be routinely conducted for all flasks throughout the 96-hour course of this assay.

The authors do not agree that additional cell count determinations should be routinely conducted. However, the authors do not disagree with the suggestion to increase the required negative control cell concentration (i.e., negative control cell count must be at a certain concentration in order for the final result to be considered valid).

Criticism: This assay should include multiple species for a more definitive toxicity assessment.

The authors somewhat agree with the multiple species concept i.e., the soil eluate would be tested using more than one algal species. However, this approach would still represent a different assay from the one currently being evaluated and it would produce a different final test result. For each new assay component added to the total procedure, there will be a corresponding increase in the complexity (interpretation) of the total assay response to a given concentration of a given pollutant(s).

Criticism: An EC_{50} tabulation should be routinely required for the positive control (zinc chloride) instead of using the single concentration currently directed by the method protocol.

This suggestion was made as part of the final procedure review that included a review of the completed single laboratory test data. The authors agree with this criticism and, while this already accomplished evaluation cannot make the correction, future use of this procedure should include EC_{50} determinations for the positive control.

Criticism: The addition of nutrients should be the same for each dilution flask. The reviewers acknowledged that, even if the suggestion was used, the various volumes of eluate might still cause (depending on what elements were leached into the original eluate) an unequal nutrient environment between the different dilution flasks.

This criticism represents a reasonable observation, but as the reviewers acknowledged, neither approach will completely solve the possibility of unequal assay environments due to the differences in soil eluate volume. Allocation of nutrients to the dilution flasks was another step for which a complete consensus was never really achieved. The basic concern of such suggestions has typically been the possibility of nutrient limitations for the 80% eluate flask or the reverse of this condition (if a more concentrated nutrient preparation was added to only one dilution) which might cause either toxicity or formation of biologically unavailable complexes and precipitates due to elements already present in the original leachate.

The 20% nutrient addition is apparently a sufficient supplement for algal growth during the 96 hour assay interval. The 90% nutrient addition and the 99% nutrient addition were not of similar concern when nutrient criticisms were presented. The original EPA Corvallis protocol selected the "proportionate nutrient addition" approach and, since a total consensus was not achieved, we have continued with this phase of the Corvallis procedure.

Criticism: Perhaps some additional testing should have been conducted as part of the ruggedness phase of this evaluation.

The authors somewhat agree that, especially in the case of this particular assay, a more extensive ruggedness evaluation (or maybe a series of method optimization studies) would probably have been beneficial especially if it would have contributed to a more enthusiastically endorsed consensus procedure.

Criticism: The method protocol should be revised to direct that valid test responses also be tabulated (and presented) for biostimulatory effects.

The authors do not disagree with the fact that biostimulatory effects can lead to environmental damage. Perhaps stimulatory effects should be quantified which would probably require that an additional control be added (the response to which would be a known increase in cell concentration as compared to the negative control). It might also be necessary to confirm the precision capability for a biostimulatory response as has been done for inhibitory responses. At present, the assay procedure does not include such a control nor does the procedure direct that stimulatory responses be recorded other than as a no observed inhibition for the tested soil sample.

Criticism: A test design of only three concentrations (dilutions) with two replicates per concentration is inadequate to allow for an EC_{50} precision estimate (during routine method application).

It should be emphasized that the procedure, as currently directed in the method protocol, represents only one valid test response. More than one valid test result would be necessary when providing a precision estimate.

Criticism: The method protocol should be separated into two component procedures and the components should be evaluated separately in terms of single laboratory capabilities. The first procedure should address the preparation of an eluate from the collected soil sample (i.e., transfer of pollutant compounds, humic substances, chemical elements, etc. from soil to leachate) and the second procedure should address all steps involving Selenastrum capricornutum. Precision, accuracy, sensitivity, etc. determinations should be acquired for each component procedure separately thus more clearly identifying which component was contributing the greatest amount of uncertainty.

The most significant advantage that the authors concede to this suggestion is that if one phase of the assay holds great promise and the other phase of the assay has too great an error for practical application, then a portion of the overall test could probably be combined with another improved component at a later date. The single laboratory evaluation cost might be increased somewhat due to the resulting increased requirement for chemical analyses (multiple soil and soil eluate analyses to characterize differences in pollutant transport and pollutant recovery from different subsamples of homogenized soil). Obviously, there would also be the addition of a chemical analytical error that would then need to be considered when assigning an eluate preparation error. While the authors accept the suggestion as a reasonable criticism, they think that the purpose of the overall assay is best served by having only the one basic procedure as opposed to two component procedures. In this case, the difference of opinion does not really interfere with achieving a consensus procedure.

Criticism: Another algal species should be selected because the algal species currently being used does not have a sufficient geographic distribution to be representative of freshwater algae.

The authors do not really agree with the technical basis presented for this recommendation. It should be remembered that the soil sample is initially taken to the laboratory for testing and that the algal cells are acquired commercially from the American Type Culture Collection. If a different algal species were selected, it should probably be selected based on an improved assay responsiveness and an improved assay sensitivity to the compounds most frequently encountered at uncontrolled waste site locations. Geographic distribution and results of previous study (as given by literature citations) might be secondary considerations. However, because of the poor sensitivity noted during the current evaluation, it is impossible for the authors not to at least consider the possibility of using another algal species.